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Numerous laboratory and clinical studies[1-7] conducted during the past ten years by Shorr and his associates indicate that strontium is a useful adjuvant to calcium in remineralization of the depleted skeleton.

Strontium has been found to be especially useful in metabolic derangements for which calcium alone may be inadequate or ineffective. Its use as a supplement to calcium for relief of postmenopausal, senile and idiopathic osteoporosis, Morquio's and Milkman's syndromes has been adequately established.[1-7]

Prolonged administration of strontium to a series[3] of 12 patients suffering from a wide variety of metabolic disorders characterized by demineralization of bone has demonstrated "unequivocally that strontium lactate is well retained."[3]

While individual variations may be expected, retention of strontium usually does not occur at the expense of calcium but is additive. Therefore, "when given in equal amounts, strontium is found to have certain distinct advantages over calcium (alone) for the remineralization of the skeleton."[3]

When maximum deposition of calcium has been achieved (the saturation point is reached on a calcium intake of about 1.5 to 2 Gm. per day[3]), the mechanism for strontium remains active.[3] Therefore, when calcium and strontium are administered in what appear to be optimal amounts, about 1.5 to 1.7 Gm. of each element daily, the retention of calcium and strontium together is far greater than that of calcium alone (usually twice the maximum retention or more).

Shorr and Carter[3] report that clinical evidence of improvement (subjective relief, and increased capacity for physical activity) in the osteoporotic patient corresponds to laboratory evidence of increase in retention of strontium and calcium over storage of calcium alone.

Case History: A striking case in the Shorr series was that of a 19-year-old woman with severe idiopathic osteoporosis, who had suffered more than 20 spontaneous fractures. Administration of strontium did not interfere with calcium, but rather "calcium storage increased at least twofold and combined calcium and strontium storage was four times as great as with calcium alone during the control period."[5-7]

When androgens and estrogens were administered concurrently, increase of strontium deposition equaled and occasionally exceeded the similar favorable effect produced in the retention of calcium. In some patients, additional storage is achieved with vitamin D, not over 50,000 units per day. Hence administration of strontium, with careful vitamin and hormone supplementation, may be expected to promote remineralization of the bony framework at double or even triple the rate to be achieved solely with calcium.[3]

Case History: A striking illustration in Shorr and Carter's series[5] is the case of a 65-year-old woman with postmenopausal osteoporosis, for whom metabolic studies showed an average balance of minus 177 mg. calcium on an intake of 129 mg. calcium per day. When the dietary calcium was raised to 1.5 Gm., the calcium balance rose to plus 123 mg. per day; and when 1.64 Gm. strontium was added to the same calcium intake, there was an average storage of 222 mg. strontium and 182 mg. calcium per day. When supplemented by intramuscular injection of 10,000 rat units estrogen and 50 mg. testosterone propionate, average daily balances increased to plus 256 mg. calcium and plus 241 mg. strontium during the treatment period of 35 days.

There has been no indication of toxicity in osteoporotic patients treated with strontium.[3,5-7] In a recent review, Shorr and Carter[3] state, "Except for relatively short periods over which 2.7 Gm. strontium was given daily, the standard daily dosage was 6.4 Gm. of strontium lactate (STRONTOLAC), equivalent to 1.74 Gm. of strontium. These amounts have been taken continuously for as long as four years without evidence of toxicity, as judged not only by symptomatic, but also by objective chemical and physiological indices."

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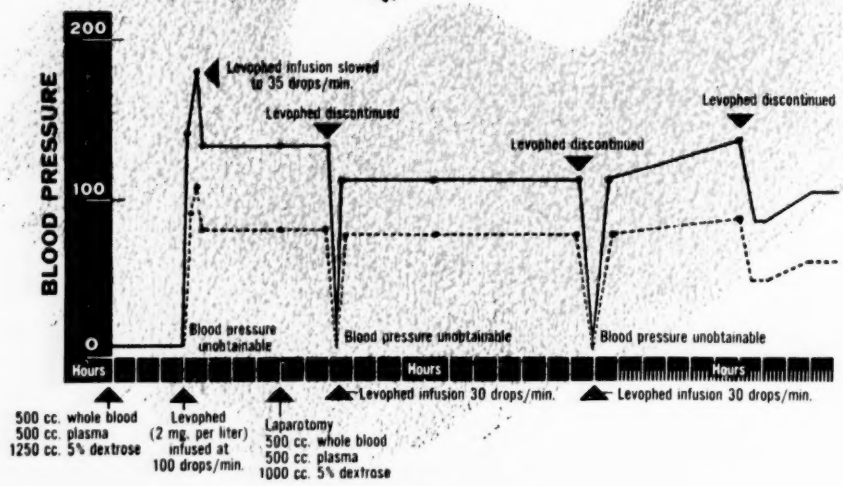

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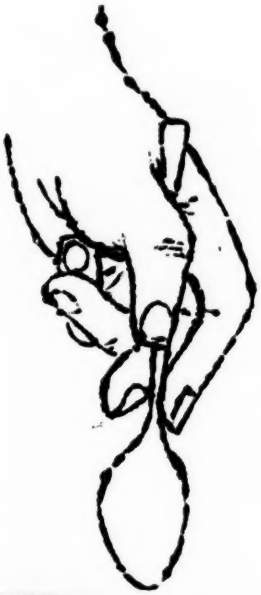

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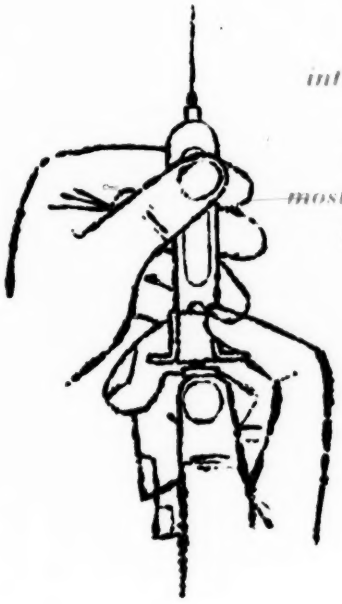

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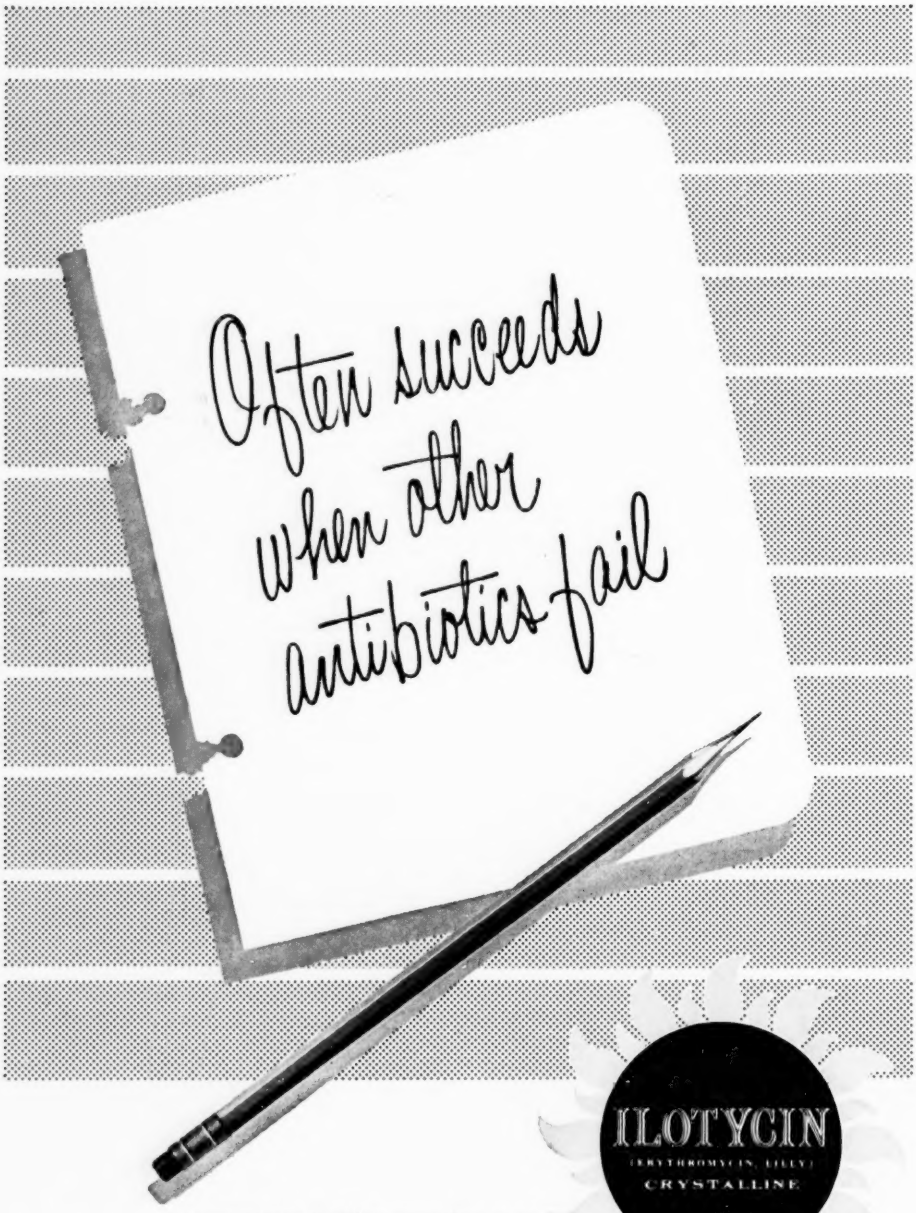

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SOME EFFECTS OF INJECTING STERILE SOLUTIONS OF STREPTOKINASE–STREPTODORNASE INTO THE SUBARACHNOID SPACE OF NORMAL RHESUS MONKEYS [1, 2]

By MORTON HAMBURGER and J. PARK BIEHL

(From the Department of Internal Medicine, University of Cincinnati College of Medicine, and the Cincinnati General Hospital, Cincinnati, O.)

(Submitted for publication December 1, 1952; accepted January 8, 1953)

Streptokinase (SK) and streptodornase (SD) solutions have been injected into the subarachnoid space of patients with meningitis, in order to promote the resolution of the inflammatory exudate (1–6). Because side reactions have been reported in certain cases (2, 3), it seemed desirable to ascertain the effect of injecting the enzymes into the uninfected subarachnoid space. Such a study was carried out in 31 rhesus monkeys,[3] along with six others in which sterile saline was substituted for the enzyme. It is the purpose of this paper to report the results of the study.

METHODS

The animals were anesthetized with pentobarbital. A cisternal tap was performed, and 2 to 3 cc. of spinal fluid removed. The enzymes were dissolved in 1 cc. of sterile saline, prepared from triply distilled water, and injected into the cisterna magna a few minutes later. Twenty-four hours afterwards, a second cisternal tap was performed. Total white and red blood cell counts, differential counts, and Pandy test were done on each sample of spinal fluid, and in some instances cultures were made. Some of the animals were then sacrificed, but others were permitted to live and were retapped at frequent intervals.

Three separate lots of enzyme solution were employed, all supplied by the Lederle Laboratories. The following concentrations were investigated: Lot 23-A: 2000 units SK plus 750 units SD; Lot 23-A: 20,000 units SK plus 7500 units SD; Lot 25-A: 2000 units SK plus 666 units SD; Lot 27-A: 2000 units SK plus 10,000 units SD.

[1] This work was done under the sponsorship of the Commission on Streptococcal Diseases, Armed Forces Epidemiological Board.

[2] Streptokinase-streptodornase was generously supplied by the Lederle Laboratories, through the courtesy of Dr. James M. Ruegsegger and Dr. Stanton M. Hardy.

[3] Monkeys used in this study were generously supplied by Dr. L. H. Schmidt, Director of the Institute for Medical Research, Christ Hospital, Cincinnati, Ohio. These monkeys had been previously used in an investigation of malaria chemotherapy. They had all been splenectomized but were otherwise entirely normal.

RESULTS

The cerebrospinal fluid response 24 hours after injection of enzyme solutions. In every instance the spinal fluid became turbid and a vigorous pleocytosis was observed. The spinal fluid counts in 87 per cent of 23 monkeys varied between 1100 and 4000, with three counts between 14,000 and 15,100. In most instances polymorphonuclear leucocytes predominated. The Pandy reaction became strongly positive.

TABLE I

Spinal fluid leucocyte count 24 hours after injection of streptokinase-streptodornase solutions into subarachnoid space of uninfected monkeys

	Dose of Enzyme (Christensen Units)			
	2000 SK 750 SD	20,000 SK 7,500 SD	2,000 SK 10,000 SD	†Sterile 0.9% NaCl
Median W.B.C. Count	1790	4700	4000	314
Range	1100–4000*	1400–14,150	3750–14,200	44–1926
Number of monkeys	10	8	5	6

SK = Streptokinase.
SD = Streptodornase.
* = 1 count was 15,100.

The effect of varying the enzyme concentration is shown in Table I. Two thousand units of streptokinase and 750 units of streptodornase in the same solution produced a median white count of 1790 per cu. mm. at the end of 24 hours. Higher concentrations of either enzyme increased the pleocytosis somewhat. Thus 20,000 units of SK and 7500 units SD gave a median count of 4700. When a solution was used which contained 2000 units of streptokinase but 10,000 of streptodornase the count was 4000. Inasmuch as the solutions were prepared in physiological saline, six monkeys were injected with 1 cubic centimeter of 0.9 per cent sodium chloride. Their median cerebrospinal fluid white cell count at the end of 24 hours was 314.

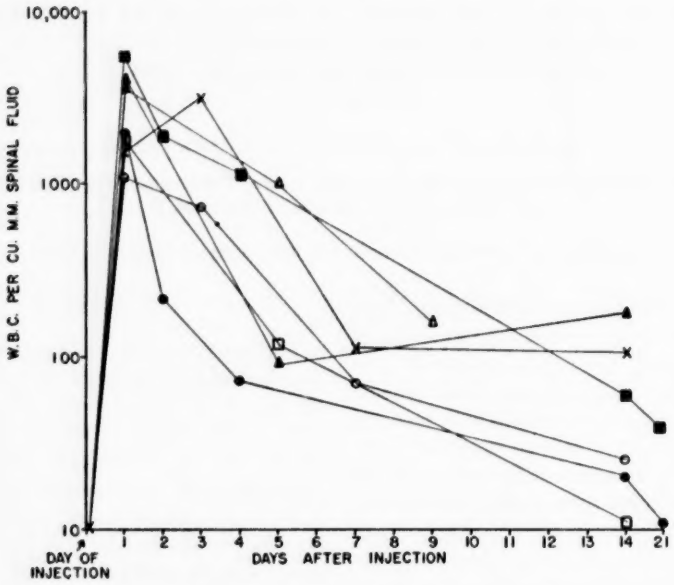

FIG. 1. SPINAL FLUID WHITE CELL COUNTS FOLLOWING INJECTION OF STERILE STREPTOKINASE-STREPTODORNASE INTO CISTERNA MAGNA OF RHESUS MONKEYS

These counts had fallen to normal within three to nine days. Thirty-six specimens of spinal fluid and surfaces of nine brains were cultured. All were sterile.

Despite these striking changes in the cerebrospinal fluid, the monkeys were not at all ill. After they recovered from the pentobarbital anesthesia, they appeared normal and alert. They ate well and exhibited no obvious neurological abnormality.

Fourteen monkeys, including representatives from each dosage schedule, were sacrificed 24 or 48 hours after injection of streptokinase-streptodornase solution. In only two animals was any gross exudate present, and in these it was minimal in extent. Most of the brains displayed slight degrees of injection of the blood vessels. Microscopic sections showed, in the majority of instances, slight degrees of polymorphonuclear reaction in the meninges. The underlying brain was never involved.

Duration of pleocytosis. Follow-up cisternal taps were performed at various intervals in six monkeys. The results (Figure 1) show that the spinal fluid white count dropped sharply from the initial level within two to four days in five of the six monkeys. By the end of a week they were all below 200 per cu. mm. By the end of two weeks they had dropped to nearly normal.

Effect of two injections on successive days. Two successive injections of 2000 units SK and 750 SD were given, 24 hours apart, to each of three monkeys. Twenty-four hours after the first injection the average spinal fluid white cell count was 2850 per cu. mm. The second injection did not increase the pleocytosis, the average count 24 hours later being 2988.

Effect of inactivated SK–SD. An enzyme solution containing 2000 units SK and 750 units SD was inactivated by heating at 56° C for 48 hours, and then injected into the subarachnoid space of five monkeys. A solution heated at 56° C for 24 hours was used in three other animals. The median spinal fluid leucocyte count was 3400 per cu. mm. with extremes of 1670 and 4750. No exudate was seen on the brain of any of the first five monkeys following sacrifice. The other three were permitted to live.

DISCUSSION

It is clear from these experiments that the injection of sterile SK–SD solutions into the unin-

fected subarachnoid space incites a vigorous outpouring of leucocytes and protein without the concomitant laying down of gross exudate in the meninges. Inasmuch as the effect was produced by inactivated as well as active enzyme, it seems likely that the spinal fluid changes were due to a non-specific protein effect. As far as we can ascertain, no reports have so far appeared in which the effect of injecting these enzymes into normal body cavities has been described.

However, Johnson (7) found that the injection of active enzyme into the pleural cavity of two patients with non-suppurative effusion led to an increase in cell count, whereas the injection of inactivated enzyme failed to do so.

SUMMARY

The injection of sterile solutions of streptokinase and streptodornase into the subarachnoid space of normal rhesus monkeys invoked a vigorous polymorphonuclear pleocytosis and a positive Pandy reaction. The white counts dropped sharply within a few days and were practically normal within two weeks. These effects were not accompanied by any sign of clinical illness in the monkeys nor by the laying down of gross exudate upon the brains of animals which were sacrificed.

ACKNOWLEDGMENTS

We are grateful to Miss Judith Carleton and Mrs. Jean Donnelly for excellent technical assistance.

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VARIATION IN THE DIURETIC RESPONSE TO INGESTED WATER RELATED TO THE RENAL EXCRETION OF SOLUTES [1]

By JACK D. ROSENBAUM, WILLIAM P. NELSON, III, MAURICE B. STRAUSS, ROBERT K. DAVIS, AND ELSIE C. ROSSMEISL

(From the Medical Service and Research Laboratory, Cushing Veterans Administration Hospital, Framingham, Mass. [Present address—V. A. Hospital, Boston, Mass.])

(Submitted for publication September 9, 1952; accepted January 21, 1953)

The administration of a large amount of water to the normal subject results in the copious excretion of a dilute urine. An extensive body of evidence indicates that this response is primarily dependent upon a decrease in the effective osmotic pressure of the plasma and extracellular fluid, with resultant inhibition of the secretion of antidiuretic hormone (ADH) by the neurohypophysis and decreased facultative reabsorption of water by the renal tubules (2–5).

Although alterations of this normal response to water loading have been studied in a variety of abnormal conditions, factors which influence the magnitude of water diuresis in normal man have received scant attention, even though Haldane and Priestley in 1916 noted that the initial high rates of urine flow which followed the ingestion of water subsequently underwent a gradual moderate decline despite a continued large fluid intake (6). Depletion of body sodium in man and experimental animals has been shown to diminish the excretion of administered water, but under the conditions of these studies general impairment of renal excretory function was evident (7–9) or was not excluded (10, 11).

The present communication describes observations which indicate that in man the maintenance of a large water load evokes a diuresis the magnitude of which is greatly influenced by factors which evoke concomitant changes in the renal excretion of solutes, particularly sodium. These influences include the dietary intake of sodium, postural effects, and the administration of various solute loads.

METHODS

The subjects were three normal men and seven adult male patients, of whom four had neurodermititis, one psoriasis, one bronchial asthma, and one rheumatoid arthritis. All were free of renal or cardiovascular disease. Dietary intake was controlled only with regard to its sodium content. The regimens employed were: *1*) "salt-free" diet providing approximately 15 mEq. of sodium daily; *2*) "salt-poor" diet providing 35–70 mEq. of sodium daily; *3*) "regular" diet of unrestricted salt content containing approximately 170–250 mEq. of sodium; *4*) "high-salt" diet consisting of the "regular" diet with 170 mEq. of added sodium chloride. The same regimen was employed for a minimum of three days before each experiment, except that the "high-salt" diet was given for only one day prior to an experiment in subjects who had previously been taking the regular diet. Repeated studies were performed on the same subjects at intervals which ranged from one week to 10 months. Hence weight changes unrelated to dietary salt intake occurred.

The subjects came to the laboratory one to two hours after breakfast, voided, and were weighed on a scale sensitive to ± 10 gm. Arterialized venous blood (12) was then obtained and a water load established by the ingestion of tap water at room temperature. In most experiments, 1500 ml. were drunk during a period of 10 to 40 minutes. The subject was again weighed and the water load was maintained throughout the experiment by oral administration of enough water after each voiding to restore body weight to its previous value.[2] In two experiments (A-1 and B-1) a larger water load of 3000 ml. was established more gradually over a period of several hours and was subsequently allowed to decrease to 1800

[1] A preliminary report (1) was presented at the Forty-second Annual Meeting of the American Society for Clinical Investigation, Atlantic City, N. J., May 1, 1950.

[2] It is recognized that with this technique there occurs a small cumulative increase in total body water, since no allowance is made for water gained by oxidation of protein, fat or carbohydrate. Furthermore, each decrement of weight on which replacement of water is based represents not only insensible water loss and water of urine and blood samples, but also the solids lost in the urine voided, the blood samples taken, and loss of weight incident to the oxidative metabolism by respiratory gas exchange. However, in experiments the duration of which never exceeded 10 hours this increase in the water load initially established is unimportant, since our own observations as well as those of Adolph (13) indicate that in man the diuretic responses to water loads ranging from 1500 to 3000 ml. are essentially the same. Moreover, we have found that administration of an additional liter of water after a load of 1500 ml. has been maintained for six hours has no apparent influence on the course of the diuretic response.

TABLE I

Renal excretion of water and solutes during maintained water loads in seated subjects

Experiment	Wgt.*	Diet†	Elapsed time‡ min.		Urine Flow ml./min.		Solute excretion rate: Na μEq./min.		K μEq./min.		Cl μEq./min.		Urea μM/min.		Urine Na mEq./L		Creatinine§ U/P		Creatinine§ Clear. ml./min.	
			I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
A-1‖	69.6	Reg	75–195	435–540	11.0	5.0	198	50	48	22	187	34			18.0	10.0	8.5	17.5	93	88
A-2	71.8	HS	60–240	420–480	16.0	10.6	458	242	132	39	461	179			28.6	22.8	5.7	8.6	91	92
B-1‖	66.7	SP	95–275	395–575	10.8	6.6	124	34			160	39			11.5	5.1	9.4	14.7	101	97
B-2	68.8	SP	30– 90	153–310	10.2	5.9	116	17	126	20	135	27			11.4	2.9	10.8	20.3	112	120
B-3	70.2	HS	60– 90	179–240	13.1	9.3	310	160	120	43	340	170			23.6	17.2	8.3	10.5	109	99
C-1	61.7	Reg	60–121	180–240	12.1	10.1	124	76	76	36	149	105			10.3	7.6	8.6	10.3	104	105
D-1	80.8	SF	77–105	333–437	9.1	6.4	18	7	85	35	30	10	238	142	2.0	1.1	11.5	18.4	105	118
D-2	81.3	Reg	80–140	200–260	12.5	10.9	103	55	53	48	107	59			8.2	5.0	7.2	9.1	90	98
E-1	70.4	SF	60–150	298–420	9.8	7.4	10	10	72	38	61	27			1.0	1.4	14.2	19.6	139	145
E-2	74.2	HS	55–174	251–400	20.6	14.8	320	195	120	68	408	217	245	207	15.5	13.2	7.0	9.8	144	146
F-1	63.3	SF	52–110	110–230	9.6	5.3	3	1	150	74	16	6	202	193	3.1	1.9	11.8	23.0	113	121
F-2	75.3	Reg	55–145	235–325	18.9	10.3	108	30	124	54	190	43	383	304	5.7	2.9	8.6	14.8	164	152
G-1	57.9	Reg	100–220	371–468	22.0	15.9	109	67	78	53	162	78	268	212	4.9	4.2	6.6	9.8	146	157
H-1	55.8	SP	82–170	261–411	12.3	9.7	93	64	63	36	120	72			7.5	6.6	9.9	12.5	123	121
H-2	54.0	SP	80–170	200–260	15.3	10.8	64	37	86	64	94	54			4.2	3.4	6.9	10.2	105	110
I-1	67.3	SP	63–120	150–210	15.2	9.4	42	14	234	92	68	24	210	268	2.8	0.7	8.9	15.1	135	142

I—Initial period of maximal urine flow.
II—Subsequent period of decreased urine flow.

* Weight before water loading.
† Reg—regular diet; HS—high salt diet; SP—salt-poor diet; SF—salt-free diet.
‡ After water loading initiated.
§ Serum creatinine determined by Peters method (15) in Subjects A, B, C and Exp. D-2; Hare method (16) in others.
‖ Initial water load of 900 ml. increased to 3000 ml. during first 240 minutes; then decreased to 1800 ml. by the end of the experiment.

ml. Except in experiments designed to study the influence of postural change, the subjects remained seated in a straight-backed chair, standing up only to void and to be weighed at intervals of approximately 30 minutes. Smoking was not permitted except in the case of Subject A, an inveterate pipe smoker, who did not inhale and who had repeatedly demonstrated that his diuretic response to ingested water was not influenced by smoking. Additional arterialized blood was obtained at intervals of one to four hours during the course of each experiment. Determinations were made of total protein, sodium, potassium, chloride, CO_2 content, creatinine, and urea in serum, as well as of hemoglobin and hematocrit. In urine, sodium, potassium, chloride, creatinine, urea, and ammonia were measured. The methods employed have been described previously (14), except that in some experiments serum creatinine was determined by the method of Peters (15) instead of that of Hare (16). Our experience confirms the finding of Hare that her procedure gives serum creatinine values in normal subjects approximately 0.2 mgm. per cent below those obtained by the techniques which measure total creatinine-like chromogen. There is good agreement between values for creatinine in urine as determined by the two methods. Therefore endogenous creatinine clearances in normal subjects based on serum values determined by the method of Peters are usually about 20 per cent lower than those based on values obtained by the Hare procedure.

RESULTS

A. Response to maintained water loads

The renal excretion of water and electrolyte during maintained water loads with the subjects seated is indicated in Table I. Initial rates of urine flow in the nine subjects ranged from 9.1 to 22.0 ml. per minute, but when the same subject was repeatedly studied while taking a relatively constant diet, the initial diuretic response was much less variable. In each of these 16 experiments the initial maximal rate of urine flow subsequently declined during the course of the experiment, although the magnitude of the water load was unchanged or actually greater at this time. Since endogenous creatinine clearance remained relatively constant, changes in flow were presumed to depend upon increased tubular reabsorption of water, also indicated by the rise in creatinine U/P ratio which took place during the course of each experiment of this group.

Initial rates of sodium excretion varied over a very wide range, consistent with the large differences in antecedent dietary sodium intake. Except when this initial excretory rate was extremely low, a large, steady decline in natriuresis was observed during the course of each experiment. This decline in excretion of sodium was relatively larger than that in water, so that concentration of sodium in the urine characteristically fell during the experimental period. Changes in chloride excretion rates and concentration were generally similar to those observed for sodium. Potassium excretion also fell strikingly in all but one instance. However, whereas excretion of water, sodium and chloride characteristically decreased steadily throughout the experiment, potassium output usually declined rapidly in the first several hours and then remained at a relatively constant level. Urea excretion, when measured, usually underwent a moderate decrease, which, similarly to potassium, often took place only in the first hours after water loading.

The regular occurrence of hemodilution both during the initial periods of maximal flow and

TABLE II

Alteration in renal excretion of water and solutes with change in posture

Experiment	Diet*	Elapsed time + min.		Urine flow ml./min.		Na μEq./min.		K μEq./min.		Cl μEq./min.		Urea μM/min.		Urine Na mEq./L		Creatinine U/P		Creatinine Clear.	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
D-1	SF	333–437	437–495	6.4	9.3	7	24	35	34	10	14	192	209	1.1	2.6	18.4	13.3	118	124
D-3	Reg	125–260	320–440	11.3	13.5	69	115	49	28	105	109	154	183	6.1	8.5	10.2	9.1	115	123
E-2	HS	251–370	370–520	15.3	13.3	209	146	72	52	235	170	197	178	13.6	11.0	9.6	11.5	147	153
F-2	HS	235–325	355–415	10.3	16.3	30	195	54	69	43	95	304	290	2.9	12.0	14.8	9.8	152	160
G-1	Reg	371–468	530–559	15.9	19.7	67	93	53	49	78	87	212	229	4.2	4.7	9.6	7.9	157	155

S—Seated.
R—Recumbent.
* SF—salt-free diet; Reg—regular diet; HS—high salt diet.
+ After water loading initiated.

TABLE III

Renal excretion of water and solutes before and after ingestion of various solutes during maintained water loads

Experiment	Period	Elapsed time*	Ingested solute†	Urine flow	Solute excretion rate Na	K	Cl	Urea	NH_3	Urine Na	Creatinine‡ U/P	Clear.
		min.	*mM*	*ml./min.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μM/min.*	*μEq./min.*	*mEq./L*		*ml./min.*
C-1	I	60–180	0	11.7	111	61	126			9.5	9.1	106
61.7 Kg.§	II	180–240	0	10.0	76	36	66			7.6	10.4	104
$NaHCO_3$	III	240–270	72	8.8	53	32	40			6.0	12.5	110
	IV	270–300	48	9.2	89	31	17			9.7	11.3	104
	V	300–360	48	11.3	208	85	14			18.4	9.7	109
	VI	360–440	48	10.6	223	100	9			21.0	9.8	104
D-4	I	90–270	0	7.9	23	37	34			2.9	11.4	90
80.4 Kg.	II	270–330	94	11.6	64	72	73			5.5	8.4	97
KCl	III	330–420	27	11.9	219	286	388			18.4	7.7	92
D-2	I	200–260	0	10.9	55	48	59			5.0	9.0	98
81.3 Kg.	II	260–410	187	12.0	74	37	75			6.2	9.0	108
NH_4Cl	III	410–500	37	13.6	120	27	140			8.8	8.1	110
I-1	I	63–150	0	14.1	35	206	187	299	13	2.5	9.4	133
67.3 Kg.	II	150–210	37	9.4	14	92	24	268	18	1.5	15.1	142
NH_4Cl	III	210–270	112	7.5	6	20	27	255	55	0.8	16.5	124
	IV‖	270–420	94	4.6‖	5	26	58	286	78	1.1	30.5	140
	V	420–480	0	12.8	47	66	126	390	66	3.7	12.1	155
B-7	I	32–88	0	8.9	53	114	116	294		5.9	10.3	92
69.0 Kg.	II	88–148	166	7.9	30	61	64	268		3.8	12.2	96
Urea	III	148–208	333	8.9	39	43	63	774		4.4	10.8	96
	IV	208–268	250	11.4	54	31	75	1343		4.7	8.8	100
	V	268–358	0	11.8	70	37	75	1196		5.9	8.1	96
I-2	I	50–146	0	8.2	5	141	25	308	9	0.6	15.1	124
66.9 Kg.	II	146–200	333	8.1	4	101	18	396	13	0.5	15.4	125
Urea	III	200–262	333	11.1	8	77	30	1195	24	0.7	10.2	113
	IV	262–320	0	8.3	4	44	24	1054	35	0.5	14.4	119
	V	320–385	0	6.9	3	45	19	949	32	0.4	16.2	112
J-1	I	85–123	0	16.3	147	115	231	293		9.0	9.8	160
72.0 Kg.	II	123–161	0	15.1	124	119	245	276		8.2	10.0	151
Urea	III	161–214	333	13.6	124	114	236	378		9.1	12.1	164
	IV	214–268	333	18.8	232	110	347	1311		12.3	8.1	152
	V	268–325	0	17.5	252	88	340	1135		14.4	8.3	146
	VI	325–377	0	16.1	235	112	323	986		14.6	8.8	142
J-2	I	139–184	0	15.2	61	161	91	375		4.0	9.9	150
74.7 Kg.	II	184–239	0	11.5	44	80	53	326		3.8	11.7	135
Urea	III	239–270	333	7.4	11	39	20	293		1.3	17.0	126
	IV	270–327	333	11.1	36	55	62	1012		3.3	11.9	132
	V	327–414	0	9.3	1	53	42	910		1.1	13.7	127

* After water loading initiated.
† Solute listed in first column.
‡ Serum creatinine determined by Peters method (15) in Subjects B, C, and D; by Hare method (16) in others.
§ Weight before water loading.
‖ Relative oliguria ascribable to nausea during the first 30 minutes of this period.

during the subsequent periods of reduced flow was indicated by a decline in hematocrit, hemoglobin and serum total protein concentrations. Concentrations of sodium and chloride in serum always declined after water loading; the fall in sodium averaged 6.3 mEq. and that in chloride 4.8 mEq. per liter. The decline in serum carbon-dioxide content during the course of the experiments averaged 1.4 mEq. per liter. Serum potassium also declined, the mean fall being 0.45 mEq. per liter.

B. The influence of dietary salt intake

Rates of diuresis were very strikingly altered when dietary intake of sodium was raised or lowered (Table I). Subject A, while taking his usual diet, excreted urine at an initial rate of 11.0 ml. per minute with a subsequent decrease to 5.0 ml. per minute. When he took a sodium chloride supplement of 170 mEq. on the day prior to study, an average flow of 16.0 ml. per minute was maintained for three hours and the rate of diuresis

never fell below 10.6 ml. per minute. A similar relationship to antecedent salt intake was demonstrated in Subjects B, D, E and F. Although in Subject F endogenous creatinine clearance was higher when salt intake was increased, the changes in urine flow were not associated with change in filtration rate in the other subjects. In every instance changes in dietary intake produced the expected increase or decrease in renal excretion of sodium and chloride. Higher rates of urine flow were associated with higher rates of sodium and chloride excretion and lower rates of flow with lower rates of salt excretion.

In each of the five subjects in whom the comparison was made, serum sodium and chloride concentrations were somewhat lower at the lower level of antecedent dietary salt intake, but the values never fell below the normal range. In four of these five subjects hemoglobin and hematocrit levels were lower with the higher salt intake.

C. The influence of change in posture

In five experiments the subjects assumed the recumbent posture after the course of the water

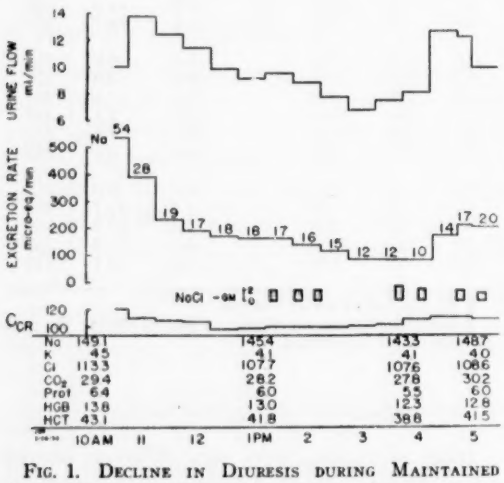

FIG. 1. DECLINE IN DIURESIS DURING MAINTAINED WATER LOAD IN SEATED SUBJECT AND AUGMENTATION AFTER INGESTION OF SODIUM CHLORIDE. EXPERIMENT B-3

Water load of 1500 ml. established and maintained throughout experiment. Numerals above sodium secretion line refer to urinary sodium concentration (mEq. per liter). Creatinine clearance (C_{CR}) in ml. per minute. Analytic data for serum electrolytes in lower portion of figure in mEq. per liter; serum protein and hemoglobin in grams per cent; hematocrit in per cent.

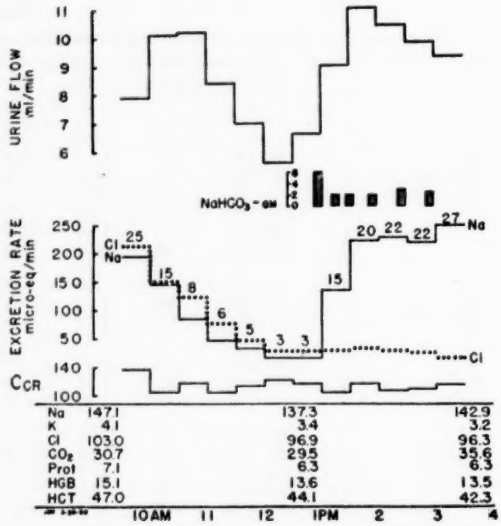

FIG. 2. DECLINE IN DIURESIS DURING MAINTAINED WATER LOAD IN SEATED SUBJECT AND AUGMENTATION AFTER INGESTION OF SODIUM BICARBONATE. EXPERIMENT B-4

Data expressed as in Figure 1.

diuresis had been observed while they sat erect for four to eight hours (Table II). Within 30 to 60 minutes after the subject reclined, increments in urine flow of from 2.2 to 6.0 ml. per minute with falling creatinine U/P ratios were observed in four experiments of the group. In the same four experiments an increase in the rate of sodium excretion and in its urinary concentration also took place. Small and probably insignificant increments in endogenous creatinine clearance were observed in three of these four studies. Little change in chloride excretion and no increase in potassium excretion accompanied the augmentation of diuresis. In the one experiment (E-2) in which assumption of the recumbent posture was associated with a further fall in sodium excretion, urine flow continued to decline.

Data pertaining to changes in the composition of blood and serum were obtained in three of the subjects, each of whom manifested during recumbency a further decrease in the level of hemoglobin, hematocrit, and serum total protein suggesting further hemodilution.

D. The influence of solute loads

In 12 experiments, after water diuresis had been established and the rate of urine flow was de-

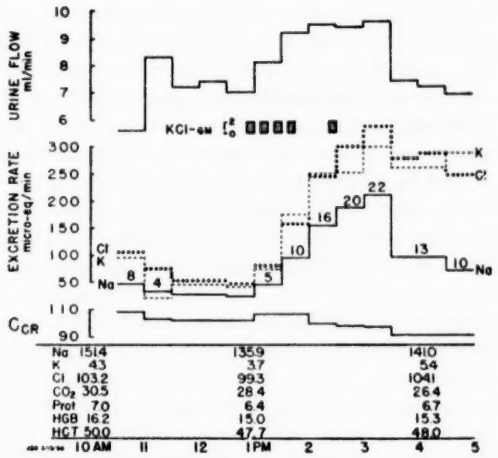

FIG. 3. AUGMENTATION OF DIURESIS AFTER INGESTION OF POTASSIUM CHLORIDE DURING MAINTAINED WATER LOAD IN SEATED SUBJECT. EXPERIMENT B-5

Data expressed as in Figure 1.

clining, the subject ingested sodium chloride, sodium bicarbonate, potassium chloride, ammonium chloride or urea (Table III, Figures 1–4). In each of these experiments urine flow increased by 2.5 to 5.4 ml. per minute after solute administration.[3] The augmentation of diuresis was regularly associated with an increase in the excretion rate and urinary concentration of sodium, except in one of the four experiments in which urea was taken.

In each of two experiments, following ingestion of sodium bicarbonate, the excretion of potassium rose, while that of chloride fell or remained essentially unchanged. After potassium chloride was given there was a sustained increase in the output of potassium, chloride, water and sodium in one experiment (D-4, Table III). In the other (Figure 3) the excretion of water rose while there was concomitant natriuresis; thereafter, sodium excretion, and with it urine flow, again declined despite continued high rates of potassium and chloride excretion. The diuretic response to urea loading, studied in four experiments, was quite variable despite the fact the increments in the excretion of this solute were consistently very large. For example, in experiment I-2, in which sodium excretion was very low, urine flow rose only during the first hour of augmented urea output, whereas in experiment J-1, with much higher rates of sodium excretion, urea loading was followed by a sustained increase in urine flow associated with a marked rise in the excretion rates of sodium and chloride.

No consistent changes incident to solute loading were noted in levels of hemoglobin, hematocrit, or serum total protein concentrations. The expected changes in serum electrolyte concentration were noted after administration of sodium bicarbonate, potassium chloride and ammonium chloride.

[3] The increase in Experiment I-1 was taken as 5.3 ml. per minute above the flow of 7.5 ml. per minute observed in period III since an acute antidiuretic response, presumably related to abdominal discomfort and nausea, occurred during period IV.

DISCUSSION

Since the early observations of Haldane and Priestley (6), little attention has been paid to variations in renal excretion of water when an excess is maintained in the body, presumably because most investigators have been concerned with the response to the administration of a single large water load. Brun, Knudsen and Raaschou (17) observed that when normal man drank water at a rate of 10 to 20 ml. per minute while recumbent

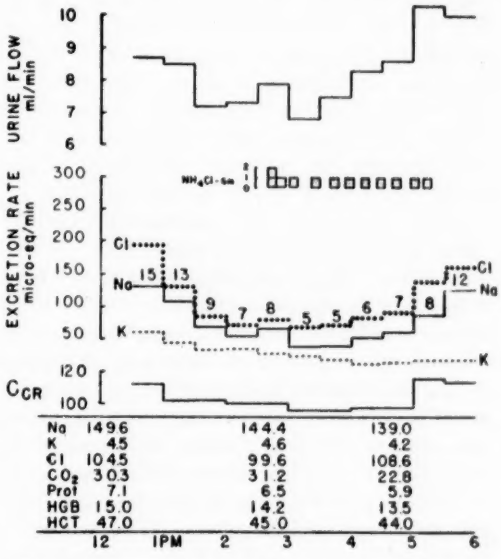

FIG. 4. AUGMENTATION OF DIURESIS AFTER INGESTION OF AMMONIUM CHLORIDE DURING MAINTAINED WATER LOAD IN SEATED SUBJECT. EXPERIMENT B-6

Data expressed as in Figure 1.

the rate of diuresis varied considerably during the first four hours. The data of Baldes and Smirk (10) demonstrate that subjects who have been depleted of sodium exhibit an impaired diuretic response to ingested water, an observation which was confirmed and extended by studies both in man and rabbits by McCance (7–9), who also demonstrated considerable reductions of glomerular filtration rate and urea clearance during salt deficiency. Impaired diuretic responses to ingested water have also been described in dogs and rats depleted of sodium (11), although Cizek and Huang (18) observed normal water diuresis in salt-deficient dogs after an initial period of impaired diuretic response associated with a decreased rate of glomerular filtration. Oliguria may also occur when water intoxication is produced in dogs (19) or rabbits (20).

However, changes in water diuresis under abnormal conditions of salt depletion or water intoxication appear to bear little relation to the variations observed in normal subjects. Changes in urine flow bore no consistent relationship to serum sodium concentration. The clearances and U/P ratios of creatinine in the present study indicate that the variations in urine flow observed cannot be related to changes in glomerular filtration rate, but, unless tubular secretion of water is postulated (21), must be determined by alterations in tubular reabsorption of water. Moreover, the data indicate that these changes in tubular reabsorption are associated with alterations in solute excretion and can be most consistently related to the concomitant rate of sodium excretion.

Except in some of the experiments which involved the administration of solute loads, the urinary solutes whose excretion rates varied most widely were sodium and chloride. Hence, urine volume would appear to vary directly with the concomitant rate of sodium excretion even if the rate of flow was actually determined by the *total* number of osmols of all solutes being excreted, as has been postulated not only under conditions of osmotic diuresis in hydropenic normal subjects (22), but also in patients with diabetes insipidus (23) and in normal subjects whose ADH activity is suppressed by water loading (24). However, the experiments with various solute loads clearly show that the rate of urine flow is not determined by the concomitant rate of total solute excretion, calculated as urea plus twice the sum of sodium, potassium and ammonia. In Experiment I-1 (Table III) urine flow rose from 9.4 to 12.8 ml. per minute after ammonium chloride ingestion, while total solute excretion increased from 518 to 775 micro-osmols per minute with urinary concentrations of 57 and 61 milli-osmols per liter. An entirely different relationship obtained in Experiment I-2, in which urea was the loading solute. Although total solute excretion increased from the pre-loading value of 610 to an average of 1244 micro-osmols per minute in the three periods after urea loading, urine flow increased only during the first of these periods and total osmolar concentration rose from 76 to a maximum of 161 milli-osmols per liter. Similar dissociation of urine flow and total solute excretion is apparent in Experiment J-2 in which urea loading increased total solute excretion to rates greatly in excess of the pre-loading values without restoring the initial high rates of flow.

The special importance of sodium excretion is apparent in these urea-loading experiments. In Experiment J-1 the rate of sodium excretion, initially 124 to 147 mEq. per minute, almost doubled following urea ingestion, and a large sustained increase in urine flow occurred. In experiment B-7, although the initial rate of sodium excretion was lower, a moderate rise took place after urea administration and a sustained rise in urine flow occurred. These experiments may be contrasted with I-2 and J-2 in which sodium excretion remained low and augmentation of urine flow after urea administration was less well maintained.

Augmentation of flow after administration of sodium bicarbonate or ammonium chloride was similarly associated with an increase in the excretion rate of sodium. The association of the excretion of water with that of sodium but not with that of potassium or chloride after administration of potassium chloride was apparent in Experiment B-5 (Figure 3) as well as in a similar study reported previously (25).

It seems clear that the decline in urine flow during maintained water loads, as well as the variations in the magnitude of water diuresis, bears no consistent relationship to the excretion of potassium, chloride, or urea but appears to be most often associated with corresponding variations in the

rate of sodium excretion. Observations which indicate that a similar relationship obtains in the diurnal variation of the diuretic response to water loading have been reported previously (25) and are consistent with studies described by Stanbury and Thomson (26). Correlation between rates of urine flow and excretion rates of chloride or sodium during water diuresis has also been recognized by Halmágyi (27), Lauson (24) and Ladd (28), as well as by Black and Thomson in subjects maintained on a constant diet with a daily fluid intake of one liter daily (29). It must be emphasized, however, that we have observed large changes in sodium excretion with small changes in water excretion and *vice versa*. Moreover, in subjects whose sodium intake is very low, initial low rates of urine flow may decline without further decrease in the very low initial rates of sodium excretion. Conversely, recumbent subjects given water loads at night characteristically manifest a steady decline in sodium excretion, without change in the rate of water diuresis (25). Welt and Nelson (30) in five experiments could find "no evident correlation between . . . the initial rate of excretion of sodium . . . and the height of the maximal diuresis attained." However, in only one of their three subjects is it possible to compare maximal water diuresis at two different levels of sodium excretion. This subject attained a flow of 18.4 ml. per minute with a creatinine U/P ratio of 6.5 while excreting 138 μEq. of sodium per minute. On another occasion while excreting sodium at a rate of 216 μEq. per minute his maximal flow was 20.1 ml. per minute and his creatinine U/P ratio attained the remarkably low value of 4.5.

The concept of "osmotic diuresis" has been extended by Ladd (28) and by Homer Smith (5, 31) to embrace the view that not only under hydropenic conditions but at any given level of ADH activity the fraction of filtered water excreted by the kidney is determined by the concomitant excretion of solute. It is postulated that in the absence of ADH activity there exists a constant "free water clearance," approximating 13–14 ml. per minute in normal man, which is in turn dependent upon a relatively constant maximal capacity for sodium reabsorption by the distal tubule (Tm^d_{Na}). Urine flows above this basal rate are believed to represent the addition of isotonic proximal tubular fluid, the sodium concentration of which approximates that of glomerular filtrate. This hypothesis requires that urine flow and sodium excretion vary together in a constant and quantitatively predictable manner. In our experiments, although an association between excretion of water and that of sodium is usually apparent, their quantitative relationship is quite variable and therefore calculated "free water clearance" also varies considerably. Such fluctuations in free water clearance imply either that the postulated reabsorptive maximum for sodium undergoes large and rapid alterations, or that facultative tubular water reabsorption is occurring to a variable extent, presumably because the completeness of ADH suppression is variable. If the objections which have been raised by others (32, 33) to the existence of a relatively fixed distal tubular maximum for sodium reabsorption are valid, to hypothesize a constant free water clearance when ADH activity is absent or minimal can have little meaning. Our data could then be interpreted to indicate that increased tubular rejection of solutes, particularly sodium, permits increased excretion of water, but that these relationships are too complex or too variable to permit precise quantitative definition.

Alternatively, it may be argued that under the conditions of our experiments small but variable amounts of ADH activity were present, at least when diuresis decreased, with resultant changes in facultative reabsorption of water. To be sure, the subjects were in a state resembling functional diabetes insipidus, ascribable to inhibition of ADH secretion by maintained hypotonicity.[4] However, it has been repeatedly demonstrated that the supraoptico-hypophyseal mechanism responds not only to changes in tonicity of the body fluids, but also to other stimuli, including alterations in the volume of extracellular fluid (14, 34–38). Except in those experiments during which sodium salts were taken, with continued loss of extracellular electrolyte, some decrease in total ECF volume

[4] Although Baldes and Smirk (10) suggested that adaptation to prolonged depression of osmolarity occurs, that such an event did not take place in our experiments is indicated by the failure of additional water loads to increase urine flow once it had diminished.

can be assumed.[5] Moreover, redistribution of ECF undoubtedly occurs with pooling of fluid in the legs as the sitting posture is maintained. Movement of extracellular electrolyte into the water introduced into the intestinal tract, first postulated by Priestley (39), would account for a further decrease in interstitial volume. Such a decrease in "effective ECF volume" (40) owing to shrinkage and redistribution of the total ECF might well serve as a stimulus for secretion of some ADH. This stimulus might be especially marked when the ECF volume was initially somewhat low owing to antecedent restriction of dietary salt. Conversely, salt loading or assumption of the recumbent posture should oppose such antidiuretic stimuli. The changes in volume tending to increase or decrease water excretion would be expected to favor parallel changes in the renal excretion of sodium (41). Similar parallelism in the excretion of salt and water has been observed in studies concerning their diurnal variation (25, 26, 42, 43).

It is possible, therefore, that altered facultative reabsorption of water accounts for some of the changes in urine flow in water-loaded subjects. If the lower rates of diuresis were always dependent upon ADH secretion, it would be anticipated that at lower rates of flow, urinary concentrations of sodium and chloride would usually increase. Actually, the concentration of salt was characteristically *lower* at lower rates of urine flow and rose when diuresis was augmented. Nor could an inverse relation between the flow of urine and its *total* osmolar concentration be demonstrated after urea loading, when osmolarity was calculated either from urinary solutes determined individually or from measurements of freezing point.[6] Moreover, patients and experimental animals with diabetes insipidus exhibit similar variations in urine flow related to the solute load presented for excretion (21, 23, 44–49).

[5] Calculation of change in ECF volume from chloride balance and concentrations of chloride in ECF is unsatisfactory since the magnitude of change is too small to be estimated in this manner. Also, as pointed out by Welt and Nelson (30) such an attempt at quantitation is invalidated by the presence of an indeterminate amount of chloride in the unabsorbed water contained within the intestinal lumen.

[6] The determinations of freezing point depression were generously performed by Dr. Alexander Leaf.

The variety of changes observed in the renal excretion of water may thus be related both to alterations in solute load and to slight variations in the level of ADH activity, dependent upon changes in "effective" ECF volume. Parallel alterations in excretion of water and of sodium would be explained if *a*) volume changes which stimulate ADH excretion also serve as stimuli for sodium retention and *b*) tubular water is obligated differently by various solutes and the obligation imposed by sodium is usually more important than that of other solutes, such as urea.

SUMMARY AND CONCLUSIONS

1. Variations in the diuretic response to ingested water were studied in 10 subjects in whom water loads were established and maintained over periods of four to nine hours.

2. When the subjects remained seated, the rates of urine flow and of sodium excretion characteristically declined during the course of each experiment; when the subjects subsequently reclined, augmentation of urine flow and sodium excretion was observed in four of five experiments.

3. The magnitude of diuresis was increased when the dietary intake of salt was large and decreased when antecedent salt intake had been restricted.

4. The ingestion of sodium chloride, sodium bicarbonate, potassium chloride, ammonium chloride, or urea was associated with an augmentation of urine flow and of solute excretion.

5. The changes in urine flow were related to variations in renal tubular reabsorption of water rather than to glomerular filtration rate.

6. The changes in urine flow were most consistently, although not invariably, related to parallel alterations in excretion of sodium.

7. It is suggested that alterations in the magnitude of water diuresis may be related to changes in sodium excretion because:

a) Despite continued hypotonicity, contraction of effective extracellular volume evokes minimal ADH activity as well as decreased sodium excretion.

b) Tubular water is obligated differently by various solutes and the obligation imposed by sodium is usually more important than that of other solutes.

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The technical assistance of Miss Miriam Halpin, Miss Helen C. Lichwell, and Miss Regina McLean is gratefully acknowledged.

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STUDIES ON COPPER METABOLISM. IX. THE TRANSPORTATION OF COPPER IN BLOOD[1]

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The isolation and identification of the copper protein from serum have clarified the problem of the nature of serum copper (1, 2). This blue protein, ceruloplasmin, is an α-globulin with a molecular weight of approximately 151,000 and contains eight atoms of copper. Ceruloplasmin has true oxidase activity and since it acts on paraphenylene diamine and polyphenols but not on monophenols and monoamines, it has been classified as a laccase (3, 4). Most if not all of the copper in normal human or pig serum is stated to be present in the form of this blue protein. The copper in serum obtained from pregnant women and from patients with infections and other diseases, like ceruloplasmin, is precipitated by 50 per cent saturation with ammonium sulfate. Furthermore, the ability of serum to oxidize paraphenylene diamine is proportional to the copper content not only in normal serum but also in serum obtained from the umbilical vein, from pregnant women and from patients with infections (5). Presumably then, in patients with hypercupremia the copper is in the form of ceruloplasmin.

The problem still remains as to whether or not ceruloplasmin serves the function of transporting newly absorbed copper from the gastro-intestinal mucosa to the liver. Since the concentration of copper (ceruloplasmin) in normal human serum is relatively constant and is not influenced by meals or by the ingestion of copper (6–8), it seems unlikely that this protein is concerned in the active transport of copper. More important is the observation (1) that the copper in ceruloplasmin does not react directly with sodium diethyldithiocarbamate, whereas copper added to serum *in vitro* does react directly with this reagent (9). This would indicate that, unlike the ability of serum to bind iron, there is no reserve capacity of normal serum to bind additional amounts of copper in the form of ceruloplasmin. It would also seem unlikely that newly absorbed copper would be transported to the liver in the form of an active enzyme.

It has been demonstrated (10) that the crystalline β_1 metal-binding globulin (transferrin) isolated from plasma interacts with copper at pH 7.4 although maximal binding occurs at pH 8.5. At pH 7.4 iron readily replaces copper and copper cannot replace iron. It has been suggested that this protein may be concerned in transporting newly absorbed copper to the liver (10). Observations made by Holmberg and Laurell (1), however, suggest that the metal-binding protein does not function in the transportation of copper.

The purpose of this paper is to present data concerning the type of binding of copper added both *in vivo* and *in vitro* to plasma of normal and abnormal human subjects as well as to that of rats, pigs and dogs. Data are also presented on the relation of erythrocyte copper to plasma copper.

This work has been in progress since 1949 and two preliminary reports have been published (8, 11).

METHODS

Plasma and red cell copper determinations were performed by the method developed in this laboratory (12). The copper in plasma which reacts with sodium diethyldithiocarbamate when this reagent is added directly to plasma is referred to as the "direct-reacting" copper fraction. The difference between the total plasma copper determined following the liberation of the copper from the protein by acid and the direct-reacting copper is referred to as the "indirect-reacting" fraction.

The direct-reacting fraction of plasma copper was measured by adding 0.5 ml. of a saturated solution of sodium pyrophosphate and 2.5 ml. of redistilled water to 1.0 ml. of plasma. The optical density (D1) was measured with a spectrophotometer (Beckman Model DU or B with cells having a 1.0 cm. light path) at a wave length of 440 mμ. Two-tenths ml. of a 0.1 per cent aqueous solution of sodium diethyldithiocarbamate was then added

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[2] National Research Council Fellow in the Medical Sciences, 1949–1951.

and the optical density (D2) was again read after a suitable time interval. A reagent blank consisting of 3.5 ml. water, 0.5 ml. of pyrophosphate and 0.2 ml. of carbamate was prepared and the optical density (D3) determined. The μg. of direct-reacting copper per 100 ml. of plasma were calculated from the formula:

$$D2 - (D1f + D3) \times K \times 100$$

where f is

$$\frac{\text{volume before addition of carbamate}}{\text{volume after addition of carbamate}} = 0.952$$

K is the constant derived from the standard curve prepared as described previously (12).

The pH of the final reaction mixture was approximately 8. Dog and pig plasma required less than five minutes for maximal color development after the addition of carbamate while human and rat plasma required 20 minutes.

Dialysis of plasma or red cells was carried out in cellophane tubing (1 cm. diameter) which had been treated previously with dilute acid and thoroughly rinsed with redistilled water. Five ml. of plasma or red cells of known copper content were pipetted into a suitable length of tubing tied at one end. After tying the other end, the tubing was suspended in a relatively large volume of an appropriate buffer and allowed to dialyze with occasional agitation for 18 hours at 2° C. The dialysate was changed twice during the 18 hours. Dialysis for a greater period of time did not result in the further removal of copper. After dialysis the volume of the sample in the tubing was measured and the copper content of the dialyzed red cells or plasma was determined.

Copper was added to plasma *in vitro* in the form of a solution of cupric sulfate. When copper sulfate was administered intravenously to dogs, a solution containing 1 mg. of copper per ml. was injected into the jugular vein. Two hundred and fifty mg. of Cupralene [3] dissolved in 150 ml. of water were administered orally to fasting human subjects. For the intravenous injections of copper in human subjects, 100 mg. of Cupralene were dissolved in 15 ml. of sterile physiologic saline and injected rapidly into an antecubital vein.

Adult male rats of the Sprague-Dawley strain were used in this study. One-tenth ml. of turpentine (Rexall Rectified Oil of Turpentine, U.S.P.) was injected in order to produce a sterile intramuscular abscess (13). Weanling rats were made deficient in copper by feeding a condensed milk diet supplemented with iron (14). Blood was withdrawn from the abdominal aorta and potassium oxalate was used as an anticoagulant.

RESULTS

Plasma copper. Total plasma copper and direct-reacting copper measurements were made on human, rat, dog and pig plasma and the results are presented in Table I.

[3] Allycuprothiocarbamide containing 19.93 per cent copper.

TABLE I

Total and direct-reacting plasma copper

Species	Human		Rat		Dog	Pig
Condition	Normal	Pregnant	Normal	Turpentine-injected	Normal	Normal
Number	7	8	10*	10*	7	2
Plasma Cu μg./100 ml.	110	228	107	208	77	172
Direct-reacting Cu μg./100 ml.	4	18	1	3	9	72
% of total Cu reacting directly	4 (0–17)	8 (0–17)	1	1	12 (0–27)	42 (40–43)

* Pooled specimens.

In normal human plasma approximately 4 per cent of the plasma copper reacted directly with sodium diethyldithiocarbamate at pH 8.0. In hypercupremic plasma of pregnancy the absolute amount of direct-reacting copper increased as well as the per cent of the total. However, 85 per cent of the additional copper in this plasma was in the indirect-reacting fraction. In plasma from normal rats, 1 per cent of the copper was in the direct-reacting fraction. In plasma with a high total copper content obtained from rats in which sterile turpentine abscesses had been produced, a similar amount of the total copper reacted directly. In

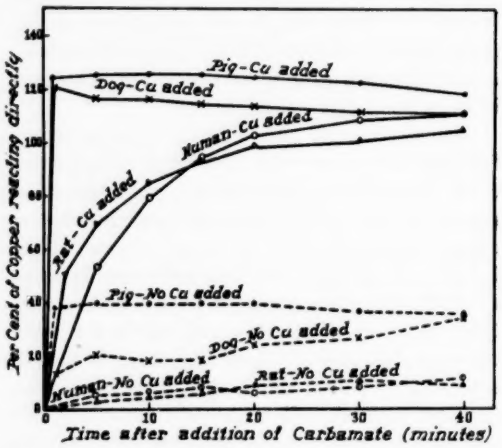

FIG. 1. THE RATE OF REACTION OF CARBAMATE WITH DIRECT-REACTING COPPER IN THE PLASMA OF SEVERAL SPECIES BEFORE AND AFTER THE ADDITION OF COPPER IN THE FORM OF COPPER SULFATE

The values for the plasma with no added copper are expressed in per cent of the total plasma copper. The values for the plasma with added copper are expressed in per cent of added copper.

plasma from normal dogs and pigs the direct-reacting fraction was higher than in man and rats, being 12 and 42 per cent of the total copper, respectively.

The rate at which the copper combines with the carbamate reagent was studied in human, rat, dog and pig plasma. As can be seen in Figure 1, the reaction rate varied markedly between the species. The rate was slowest in human and rat plasma and most rapid in pig plasma.

Copper added to plasma in vitro. Amounts of copper ranging from 100 to 800 μg. per 100 ml. were added ***in vitro*** to human, rat, dog and pig plasma and measurements were made of the reactivity of the copper added. The results are presented in Table II.

In human, dog and pig plasma all of the copper added could be accounted for in the direct-reacting fraction. This was true for both normal human plasma and hypercupremic pregnancy plasma and was independent of the amount of copper added or the initial level. In all three species, the direct-reacting fraction was greater than the amount of copper added. This discrepancy was consistent and was greater than could be accounted for in the error of the method. Thus it would appear that the addition of copper caused a portion of the original indirect-reacting copper to react directly. In normal and hypercupremic rat plasma this influence of added copper was not observed. Only 82 to 96 per cent of the added copper reacted directly.

To determine if plasma depleted of copper is capable of binding added copper so that it will not react directly with carbamate, copper was added to plasma obtained from copper-deficient rats. In spite of the fact that the copper level in such plasma was reduced from the normal of 107 μg. per 100 ml. to 18 μg. per 100 ml., 90 to 102 per cent of the copper added reacted directly with the carbamate reagent, indicating that there was no latent capacity to bind copper in the form in which most of it is bound normally in plasma.

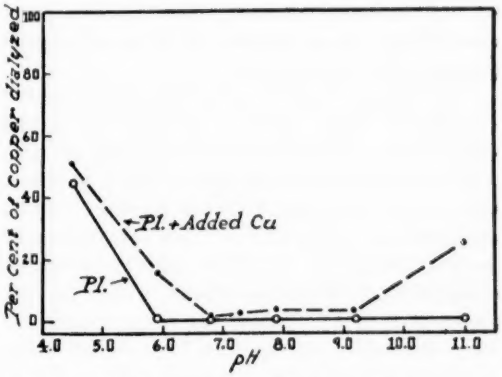

FIG. 2. THE INFLUENCE OF pH ON THE DIALYZABILITY IN 18 HOURS OF COPPER IN NATIVE PLASMA AND OF COPPER AFTER THE ADDITION OF COPPER SULFATE TO PLASMA

The rate at which added copper reacted with carbamate is shown in Figure 1. The rate was exceedingly rapid in dog and pig plasma, complete reaction taking place within five minutes. The curves for rat and human plasma are hyperbolic in shape and level off in 20 to 30 minutes. The rate was somewhat faster in rat plasma than in human plasma. In human plasma no significant difference in rate was observed when normal,

TABLE II

Total and direct-reacting plasma copper after the addition of copper in vitro

Species	Human		Rat			Dog	Pig
Condition	Normal	Pregnant	Normal	Cu-deficient	Turpentine-injected	Normal	Normal
Number	4	8	4	4	7	10	2
Plasma Cu *μg./100 ml.*	159	228	107	18	198	83	172
Cu added *μg./100 ml.*	432	430	271	246	233	424	380
Direct-reacting Cu *μg./100 ml.*	496	506	246	236	214	523	546
% of Cu added reacting directly *	114 (100–118)	113 (104–119)	91 (82–96)	93 (90–102)	91 (83–97)	121 (111–142)	125

* Calculated by subtracting the amount of direct-reacting copper in the plasma prior to the addition of copper from the amount of direct-reacting copper after the addition of copper, and dividing by the amount of copper added to the plasma.

chronic leukemia (normal plasma copper), pregnancy (high plasma copper), or other pathological plasma samples were used.

The influence of pH on the dialyzability of native plasma copper (indirect-reacting) and on the dialyzability of copper added to plasma *in vitro* (direct-reacting) was studied in order to determine if the two types of copper binding could be differentiated in this manner. The results are presented in Figure 2. It will be seen that no copper dialyzed from the native plasma within the pH range of 6 to 11. Below pH 6 the copper became dialyzable. On the other hand, copper added to plasma seemed to be more labile and was dialyzable above pH 9 and below pH 7.

Plasma copper after the oral administration of copper. Two hundred and fifty mg. of Cupralene, containing approximately 50 mg. of copper, were administered orally to each of three fasting normal human subjects. Attempts to study blood copper changes, at intervals after the ingestion of copper sulfate or copper acetate in doses of 150 to 200 mg., met with failure because of the emetic action of these inorganic compounds. The ingestion of the above dose of Cupralene was followed by nausea and mild, transient abdominal cramps, but not by vomiting. The changes in plasma and red cell copper after the administration of Cupralene are shown in Figure 3.

In none of the three subjects given Cupralene orally was there a significant change in the plasma

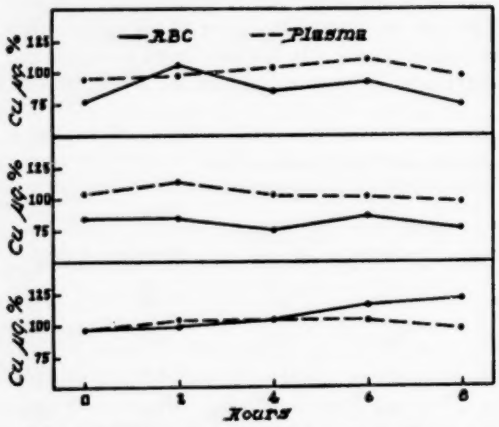

FIG. 3. PLASMA AND RED BLOOD CELL COPPER IN THREE NORMAL HUMAN SUBJECTS FOLLOWING THE ORAL ADMINISTRATION OF 50 MG. OF COPPER IN THE FORM OF CUPRALENE

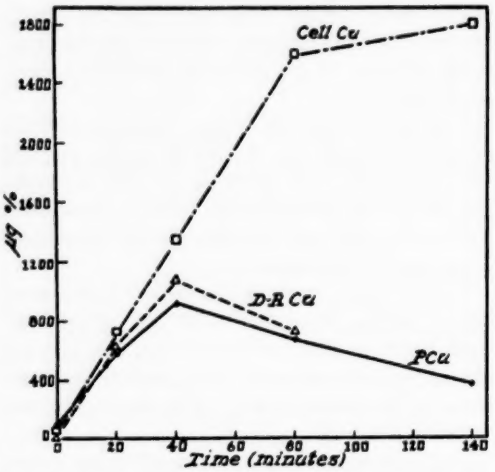

FIG. 4. TOTAL PLASMA COPPER (P.CU), DIRECT-REACTING PLASMA COPPER (D-R CU) AND RED CELL COPPER (CELL CU) IN A DOG AT VARIOUS INTERVALS OF TIME AFTER THE ORAL ADMINISTRATION OF 165 MG. OF COPPER PER KG. BODY WEIGHT IN THE FORM OF COPPER SULFATE

copper during the eight hour period of study. The erythrocyte copper values were not so uniformly stable but no significant or consistent increase in direct-reacting copper could be detected in the eight hour period.

A dog was given 165 mg. of copper per kg. of body weight in the form of copper sulfate in a single oral dose in order to study the type of binding of copper which is in the process of transportation to the liver. This amount of copper sulfate caused vomiting and death of the dog four hours later. The changes in total plasma copper, direct-reacting copper, and red cell copper are shown in Figure 4.

A considerable amount of copper was absorbed, as evidenced by the increase in the plasma copper level from 100 μg. per 100 ml. to 910 μg. per 100 ml. in 40 minutes. All of the copper appearing in the plasma reacted directly with carbamate. More surprising was the fact that the red cell copper level increased from 75 μg. per 100 ml. to 2800 μg. per 100 ml. During the period 40 to 140 minutes after the copper was given, the red cell copper continued to increase while the plasma copper level decreased from 900 to 360 μg. per 100 ml. Approximately two hours after the copper was given, 89 per cent of the copper in the blood was in the

erythrocytes and only 11 per cent was in the plasma.

Plasma copper after the intravenous administration of copper. In order to study the form in which copper is transported in blood after the intravenous administration of copper, Cupralene was administered intravenously to three normal human subjects and to one dog. Seven dogs were given copper sulfate intravenously. The results are summarized in Table III. In the human subject, 90 per cent of the copper which appeared in the plasma after the administration of Cupralene reacted directly with carbamate. In the dog, all of the copper in the plasma after the administration of copper sulfate reacted directly with carbamate. Apparently, as a result of experimental error, the mean direct-reacting copper was slightly greater than the total plasma copper.

The changes in total plasma copper, direct-reacting copper, and red cell copper at various intervals of time after intravenous administration of copper are shown in detail in Figure 5 (Cupralene;

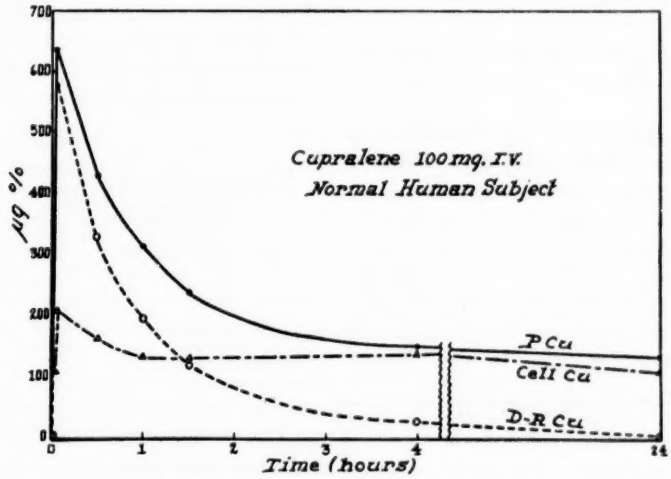

FIG. 5. TOTAL PLASMA COPPER (P.CU), DIRECT-REACTING PLASMA COPPER (D-R CU) AND RED CELL COPPER (CELL CU) IN A NORMAL HUMAN SUBJECT AT VARIOUS INTERVALS OF TIME AFTER THE INTRAVENOUS ADMINISTRATION OF 20 MG. OF COPPER IN THE FORM OF CUPRALENE

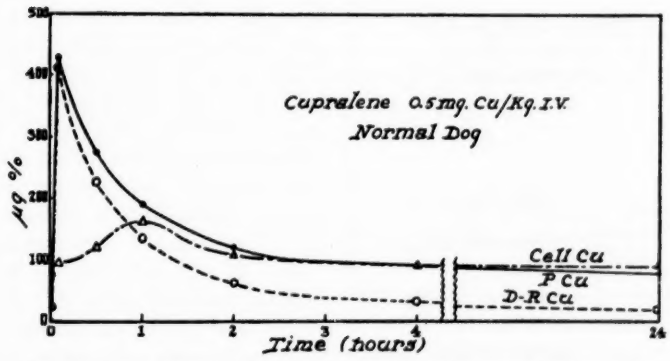

FIG. 6. TOTAL PLASMA COPPER (P.CU), DIRECT-REACTING PLASMA COPPER (D-R CU) AND RED CELL COPPER (CELL CU) IN A NORMAL DOG AFTER THE INTRAVENOUS ADMINISTRATION OF 0.5 MG. OF COPPER PER KG. BODY WEIGHT IN THE FORM OF CUPRALENE

TABLE III

Total and direct-reacting plasma copper before and ten minutes after the intravenous administration of copper

Fraction	Human		Dog	
	Before	After	Before	After
Total plasma copper μg./100 ml.	127	639	77	691
Direct-reacting plasma copper μg./100 ml.	4	578	9	737

The determinations were made in three normal human subjects after the i.v. administration of 20 mg. of copper in the form of "Cupralene"; in seven dogs after the i.v. administration of 0.5 mg. of copper/kg. in the form of copper sulfate. The values given are the means for each group.

normal human subject); Figure 6 (Cupralene; normal dog); and Figure 7 (copper sulfate; normal dog).

Following the administration of Cupralene to the human subject (Figure 5) or a dog (Figure 6), the rate of clearance of the copper from the plasma was rapid and complete in about four hours even though the initial ten minute levels were above 400 μg. per 100 ml. This rapid decrease was due to a decrease in the direct-reacting fraction. A significant increase in red cell copper was observed in both species. In man, this increase occurred within ten minutes after the injection. The red cell copper returned slowly to the baseline value during the first 24 hours. In the dog the red cell copper increase was maximal one hour after the injection and returned to the baseline value within three hours after the injection. The rate of removal from the plasma of intravenously injected copper sulfate was more rapid than the removal of Cupralene. In the dog, following the administration of copper sulfate the plasma copper level rose above 700 μg. per 100 ml. and decreased to near the baseline value within 30 minutes. There was a considerably greater and earlier increase in red cell copper following the administration of copper sulfate to dogs than following the administration of Cupralene. Thus

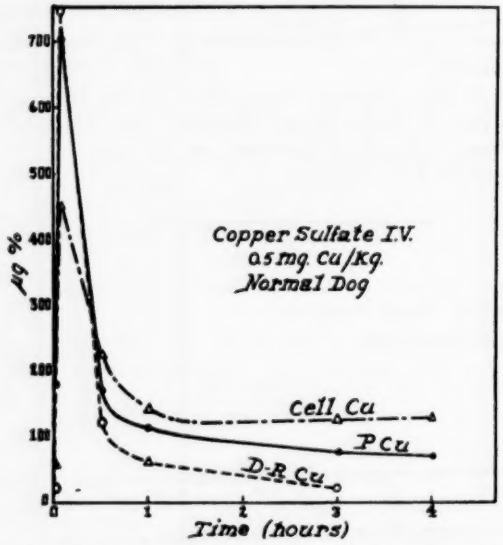

FIG. 7. TOTAL PLASMA COPPER (P.Cu), DIRECT-REACTING PLASMA COPPER (D-R Cu) AND RED CELL COPPER (CELL Cu) IN A NORMAL DOG AFTER THE INTRAVENOUS ADMINISTRATION OF 0.5 MG. OF COPPER PER KG. BODY WEIGHT IN THE FORM OF COPPER SULFATE

TABLE IV

The reactivity with sodium diethyldithiocarbamate of copper added to bovine serum albumin and to human β_1 metal-binding globulin as compared with copper added to normal human serum

	Cu added μg./100 ml.	Direct-reacting Cu μg./100 ml.	% of Cu added reacting directly
Bovine albumin *	342	310	91
Human β_1 globulin *	342	305	88
Human serum	456	478	104

The measurements were made 15 minutes after the addition of copper.
* 2.5 per cent solution (wt./vol.).

the red cell membrane appears to be more permeable to this inorganic compound than to the organic compound, Cupralene.

Copper albumin and copper β_1 metal-binding globulin. Since it has been suggested that copper may be transported by being bound to albumin (15) or to the β_1 metal-binding globulin (10), and since the data presented above indicate that copper added to plasma is not bound in the same manner as the native plasma copper, the combination of copper with bovine serum albumin and human metal-binding globulin (Cohn fraction IV-7) was studied. The results are presented in Table IV. Eighty-eight to 91 per cent of the copper combined with either of these proteins reacted directly with carbamate.

The transfer of copper between cells and plasma. When copper is added to plasma and the plasma is incubated with red cells, there is an appreciable movement of copper into the red cells.

TABLE V

The partition of copper between plasma and red cells after the addition of copper

Increasing quantities of a solution of copper sulfate (1000 μg./ml.) were added to 10 ml. aliquots of pooled rat blood (V.P.R.C., 48 ml./100 ml.). The mixture was incubated at 37° C for four hours. The results are expressed in μg. of added copper recovered in the plasma and the cells. The failure of columns (2) and (3) to equal column (1) is explained by technical variation.

(1) Cu added	(2) Plasma Cu	(3) RBC Cu	(4) % of added Cu in RBC
μg.	μg.	μg.	
18	13	3	16.6
36	30	5	13.8
91	70	13	14.3
182	163	27	14.8
364	293	50	13.8
546	451	78	14.3
728	631	106	14.6
909	792	136	15.0

This is shown in Table V. Various amounts of copper, in the form of copper sulfate, were added to whole blood (rat). The blood was then incubated at 37° C. for four hours. The red cell copper increased with increasing amounts of copper added to the whole blood. Washing the cells five times with saline did not remove the copper and the copper remained non-dialyzable even after complete hemolysis of the cell. The per cent of the copper added which migrated into the red cells remained relatively constant at about 15 and the ratio of plasma copper : cell copper remained constant at about 6 : 1 when more than 20 μg. of copper were added.

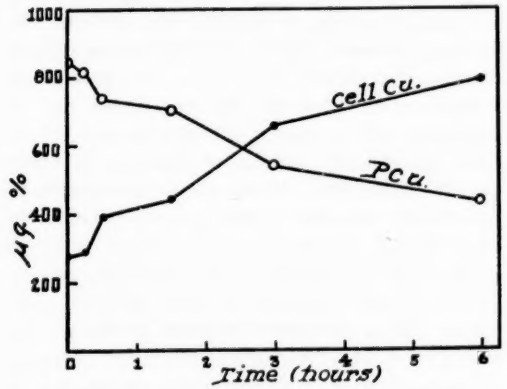

FIG. 8. TRANSFER OF COPPER FROM PLASMA TO RED CELLS DURING INCUBATION OF BLOOD

A normal dog was given 0.5 mg. of copper per kg. of body weight intravenously in the form of copper sulfate. Two minutes following the injection a sample of blood was withdrawn from the other jugular vein and incubated for six hours at 37° C.

As shown in Figure 8, when copper sulfate is injected intravenously into a dog and a sample of blood is removed and incubated at 37° C., there is a rather rapid shift of copper from the plasma into the cells. The same is true, as shown in Table VI, Exper. D, when Cupralene is injected into a human subject and a sample of blood is removed and incubated at 5° C. However, when cells containing an increased amount of copper are incubated with plasma containing a normal amount of copper (Table VI, Exper. B), there is no shift of copper from the cells into plasma. Furthermore, when normal plasma and normal cells (Table VI, Exper. A) or hypercupremic plasma (pregnancy) and normal cells (Table VI, Exper. C) are incubated, there is no shift of copper from the plasma into the cells. Thus, indirect-reacting copper does not transfer from plasma to cells regardless of the plasma copper level. Direct-reacting copper, on the other hand, transfers readily.

DISCUSSION

From the data presented it is evident that copper is present in human, rat, dog and pig plasma bound to protein in at least two different ways.

The indirect-reacting copper which constitutes approximately 96, 99, 88, and 58 per cent of the copper normally present in human, rat, dog and

TABLE VI

The transfer of copper between cells and plasma during incubation of human blood at 5° C

Experiment	Condition	Period of incubation	RBC Cu	Plasma Cu
		days	*μg./100 ml.*	*μg./100 ml.*
A	Normal cells	0	124	87
	Normal plasma	3	127	87
B	Cells with high Cu *	0	400	63
	Normal plasma	3	400	68
C	Normal cells	0	88	217
	Plasma with high Cu	3	93	230
D	Cells with high Cu	0	275	845
	Plasma with high Cu	1	790	435

* Obtained from a human subject following the intravenous administration of 20 mg. of copper in the form of Cupralene.

pig plasma, respectively, has been identified by Holmberg and Laurell (1, 2) as a copper α-globulin. It is primarily this fraction which increases in such conditions as pregnancy, acute leukemia, infections and other pathological conditions in human subjects (1, 5, 16, 17) and following experimentally induced fever in animals (14).

The remainder of the copper in plasma is in a form which reacts directly with sodium diethyldithiocarbamate but which, like the indirect-reacting fraction, is also not dialyzable at pH 7.5. The specific protein or proteins to which this fraction of the copper is bound are not known. Copper combined with serum albumin or with the β_1 metal-binding globulin is likewise not dialyzable within the physiologic pH range [4] and reacts directly with carbamate. Therefore, it is possible that the direct-reacting copper fraction in plasma is bound to either or both of these proteins. The data of Holmberg and Laurell (1) suggest, however, that the metal-binding globulin does not function in this capacity. Since one of the most interesting aspects of the chemistry of copper is its ability to form complexes and to form simultaneous links to the same organic molecules, the so-called "chelation" phenomenon (18, 19), it would not be surprising if the direct-reacting copper is bound to several different proteins rather than to a specific copper-transporting protein such as is the case with iron (10).

From our observations and from those of Holmberg and Laurell (1–5) a concept of the functional significance of these two types of copper in plasma may be derived.

It seems unlikely that ceruloplasmin (indirect-reacting fraction) serves to transport newly absorbed copper from the gastro-intestinal tract to the liver. Thus, this fraction remains relatively constant in normal human subjects and animals and is not influenced by the addition of copper to plasma or by the oral or intravenous administration of copper. Again, even in plasma of low copper content (obtained from animals deficient in copper) there is no reserve capacity to bind copper in this form. Since this copper protein has oxidase activity, it apparently represents a circulating copper-containing enzyme which is normally present in plasma and which increases in certain pathological situations.

[4] Unpublished observations.

On the other hand, following the addition of copper to plasma, or following the oral or intravenous administration of copper to dogs and the intravenous administration of copper to human subjects, there is a striking increase in the direct-reacting copper fraction. This would seem to indicate that it is this fraction which is actively concerned in the transportation of copper to the principal storage site, namely, the liver. Since normally only small quantities of copper are absorbed daily (8) and since copper is rapidly removed from the plasma, it is not surprising that under normal circumstances this fraction represents only a small portion of the total circulating copper.

The possible role of the red cell in the transportation of copper needs mention. It is obvious from the experiments reported here that when large amounts of copper are given either orally or intravenously, a significant portion of the copper is taken up by the cells. However, since the amounts of copper given far exceeded the physiologic range and since the administration of even as much as 50 mg. of copper orally in a single dose to human subjects was not associated with a significant increase in erythrocyte copper, it would seem that this mechanism is of no physiologic importance. The amount of copper in red cells is exceedingly constant in human subjects even under pathologic circumstances associated with an increase in plasma copper (6, 16).

It is interesting to consider the striking differences between plasma iron and plasma copper. Plasma iron represents iron in the process of transportation from one site to another and is combined with a specific protein, the sole or, at least, the primary function of which is to transport the iron (10). Under normal circumstances only about one-third of this protein is bound to iron (20–22) so that there is a considerable capacity held in reserve. The administration of relatively small amounts of iron orally is associated with a detectable increase in the plasma iron level (23). Iron administered intravenously is removed relatively slowly over a period of 8 to 24 hours (23).

The situation in regard to copper is quite different. Normally only a very small fraction of plasma copper represents copper in an inactive form in the process of transportation (to sites where it is to be converted into a functioning

enzyme). The greater part of the circulating copper is in the form of an active enzyme. The administration of relatively large amounts of copper orally is not associated with an increase in the plasma copper level, apparently because copper in the form in which it is bound in plasma after absorption (direct-reacting) is removed from the circulation at a rate greater than it can be absorbed.

SUMMARY

Evidence is presented that copper is present in human, rat, dog and pig plasma in at least two different forms. The fraction which reacts with sodium diethyldithiocarbamate only after removal from the protein by treatment with hydrochloric acid corresponds to the copper α-globulin (ceruloplasmin) isolated by Holmberg and Laurell. This fraction constitutes approximately 96, 99, 88 and 58 per cent of the copper normally present in human, rat, dog and pig plasma, respectively. The remainder of the copper in plasma is in a form which reacts directly with the carbamate reagent but, like the indirect-reacting fraction, is also nondialyzable within the physiologic pH range. The hypercupremia of pregnancy and that in infections in human subjects and in association with experimentally induced infections in animals, is due to an increase in the indirect-reacting fraction. The hypercupremia which follows the oral or intravenous administration of copper is due to an increase in the direct-reacting fraction.

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THE SERUM POLYSACCHARIDES IN DIABETIC PATIENTS WITH AND WITHOUT DEGENERATIVE VASCULAR DISEASE

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Complex carbohydrate substances have been demonstrated histologically in hyaline material present in the glomerular and retinal capillary lesions which develop in diabetic patients. There has been increasing speculation that the source of this material might be polysaccharides present in abnormal amounts in the blood of such individuals (1–3). Hitherto, however, the glycoproteins in the sera of diabetic patients have not been investigated systematically. A few observations suggest that abnormal concentrations of these substances occur in the serum of such patients (4), but others indicate that they are within the normal range (5, 6). Insufficient additional information is presented in these studies to allow correlation of the values observed with the absence, or presence, and extent of vascular complications.

This report is based on a study of certain polysaccharide components in the sera of diabetic patients with and without clinically detectable degenerative vascular disease. The results indicate that significant increases in the serum concentrations of some of the substances measured do occur in individuals with this complication. The possible significance of these observations is discussed.

MATERIAL

Sera were obtained from 91 patients from the wards and clinics of Montefiore Hospital, other hospitals, or from the office practice of private physicians. The subjects were free from demonstrable diseases other than diabetes, such as cancer, acute and chronic infections, and acute myocardial infarction, which are known to influence the concentration of polysaccharides in the serum (5–14). Sixty-six patients had diabetes mellitus. This group was divided further into three categories, depending on the existence and extent of associated degenerative vascular complications. Since one subgroup of diabetic patients had impairment of renal function, which may affect the serum polysaccharide levels, 10 non-diabetic individuals with renal disease were included to evaluate the effects of renal insufficiency *per se*. Fifteen subjects who had neither diabetes nor renal disease formed a control group.

Group I. Non-diabetic controls

This group consisted of 15 individuals who were seen for periodic examinations and found to be in good health. Their ages ranged from 28 to 88 years. Diabetes and renal disease were excluded by appropriate studies.

Group II. Patients with diabetes mellitus

This group was made up of 66 patients. Depending on the existence and extent of degenerative vascular complications, they were divided into three subgroups:

A. Uncomplicated

Fourteen patients, varying in age from 36 to 73 years, formed this class. Diabetes was known to have existed for 2 to 13 years with an average of about seven years. Four individuals did not require insulin. The other 10 patients required between 10 to 30 units of insulin per day. Hypertension, proteinuria, renal insufficiency, retinopathy, neuropathy, coronary artery and peripheral vascular disease were excluded by appropriate clinical, laboratory and X-ray examinations.

B. Complicated: degenerative vascular disease without hypertension, proteinuria, or renal insufficiency

There were 18 patients in this category. Their ages ranged from 27 to 83 years. The known duration of diabetes was 4 to 20 years, with an average of about 11 years. The insulin requirement of these patients varied from 0 to 120 units per day. Individuals in this category had one or several degenerative vascular complications which included retinopathy, neuropathy, occlusive coronary artery and peripheral vascular disease.

C. Complicated: degenerative vascular disease with hypertension, proteinuria, and renal insufficiency

The 34 patients in this group varied in age from 28 to 83 years, and were known to have had diabetes for 10 years or more, except for one individual in whom the known duration of this disease was eight years. These patients required 0 to 110 units of insulin daily. Individuals in this category had degenerative vascular changes similar to those found in the preceding category (Group IIB). However, in addition, hypertension and proteinuria, and, in most cases, renal insufficiency existed. A fully developed Kimmelstiel-Wilson's syndrome (15) was demonstrated in 14 individuals. The urinary sediments of the remaining 20 patients were not specifically examined for doubly refractile lipoids. Therefore, whether these 20 patients had hypertensive renal vascular disease

(nephrosclerosis) alone, or in association with diabetic glomerulosclerosis, was not settled.

Group III. Non-diabetic patients with renal disease

There were 10 patients in this group. Two individuals had acute diffuse glomerulonephritis and three had chronic glomerulonephritis. There were three patients with hypertensive renal vascular disease and renal insufficiency, one with chronic pyelonephritis, and one with renal amyloidosis.

METHODS

Twenty to 30 ml. of blood were drawn by venipuncture from subjects in the post-absorptive state and placed in a refrigerator. Within four to six hours, the sera were separated and again stored in a refrigerator until they were analyzed on the following day. Samples containing even small amounts of hemoglobin were discarded.

Total non-glucosamine polysaccharides bound to serum protein were measured by the anthrone method of Graff and his associates (16). *Serum glucosamine* was determined by the procedure of West and Clarke (6). *Mucoprotein* was isolated from the serum and analyzed for *protein* by estimating the *tyrosine content* using Folin's reagent according to the method of Winzler and his associates (17). *The non-glucosamine polysaccharides in the mucoprotein fraction of the serum* were determined by Graff's method (16). Analyses were performed in duplicate. The differences between duplicate determinations were 3 per cent or less for the non-glucosamine polysaccharides bound to serum protein, 4 per cent or less for serum glucosamine, and 5 per cent or less for the carbohydrate and protein components of the mucoprotein. The sensitivity and specificity of the isolation procedures and colorimetric reactions on which these measurements depend are poorly understood, but the results are reproducible with reasonable accuracy. Control values which we obtained are in good agreement with those previously reported by other investigators.

Fasting blood sugars were determined simultaneously on separate blood samples by the Somogyi method.

RESULTS

The values obtained for the serum polysaccharide substances measured in the various groups are summarized in Table I, and represented graphically in Figures 1, 2 and 3.

Total non-glucosamine polysaccharides bound to serum protein (Figure 1)

Values in the non-diabetic control group (Group I) ranged from 119 to 160 mg. per cent with a mean of 146 ± 11.2 mg. per cent. The levels in uncomplicated diabetic patients (Group IIA) remained in the normal range. In contrast, distinct elevations of this component were found in the complicated diabetic groups (Group IIB and IIC), and in non-diabetic patients with renal disease (Group III).

Serum glucosamine (Figure 2)

The range in the non-diabetic control group was from 84 to 116 mg. per cent, with a mean value of 97 ± 8.3 mg. per cent. Comparable values were obtained in the uncomplicated diabetic group. Again, significantly higher levels were obtained in

TABLE I

Serum concentrations of polysaccharide substances measured in the groups of patients studied

Group	Number of cases	Total non-glucosamine polysaccharides bound to protein *mg. per cent*			Glucosamine *mg. per cent*			Mucoprotein					
								Non-glucosamine polysaccharides *mg. per cent*			Protein (tyrosine) *mg. per cent*		
		Mean	S.D.*	S.E.†	Mean	S.D.*	S.E.†	Mean	S.D.*	S.E.†	Mean	S.D.*	S.E.†
I Non-diabetic controls	15	146	11.2	2.9	97	8.3	2.1	10.5	2.8	0.7	3.7	0.8	0.2
II Diabetic													
A. Uncomplicated	14	145	15.8	4.2	112	10.0	2.7	9.9	2.6	0.7	3.4	0.9	0.2
B. Complicated—without renal involvement	18	184	21.5	5.1	145	15.1	3.6	12.3	4.3	1.0	4.1	1.3	0.3
C. Complicated—with renal involvement	34	193	26.3	3.5	153	20.2	4.5	18.3	9.9	1.7	5.3	2.0	0.3
III Non-diabetic with renal disease	10	228	51.3	16.2	155	28.1	8.9	29.3	9.7	3.1	9.7	6.0	1.9

* Standard Deviation.
† Standard Error of the Mean.

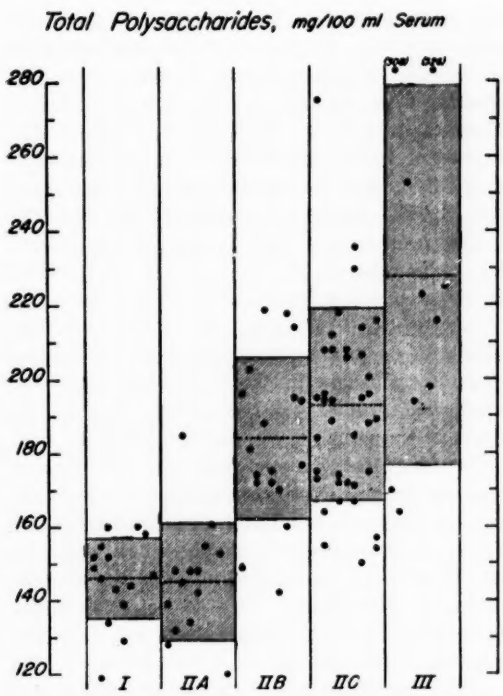

FIG. 1. THE TOTAL NON-GLUCOSAMINE POLYSACCHARIDES BOUND TO SERUM PROTEIN AMONG PATIENTS IN THE NON-DIABETIC CONTROL GROUP (I), UNCOMPLICATED DIABETIC PATIENTS (IIA), COMPLICATED DIABETIC PATIENTS WITHOUT RENAL INVOLVEMENT (IIB) AND COMPLICATED DIABETIC PATIENTS WITH RENAL INVOLVEMENT (IIC)

III designates non-diabetic patients with renal disease. The dashed horizontal lines represent the mean in each group. The shaded area encloses the mean ± S.D.

the complicated diabetic groups, and in non-diabetic patients with renal disease. As with the total non-glucosamine serum polysaccharides bound to serum protein, there was no significant difference between the concentrations observed in the complicated diabetic group with proteinuria, renal insufficiency and hypertension (Group IIC), and the complicated diabetic group without these findings (Group IIB). The average increases above normal levels were of a comparable order of magnitude in Groups IIB, IIC, and III.

Serum mucoprotein (Figure 3)

In the non-diabetic control group, the protein (tyrosine) content of the serum mucoprotein varied from 2.2 to 5.3 mg. per cent with a mean of 3.7 ± 0.8 mg. per cent. The non-glucosamine polysaccharide content was 6.1 to 14.0 mg. per cent with a mean value of 10.5 ± 2.8 mg. per cent. In the uncomplicated diabetic group and, with one exception, among the complicated diabetic patients without renal disease, these values were in the normal range. Greater variations were encountered in the complicated diabetic patients with renal involvement and in non-diabetic patients with kidney disease. In some diabetic and non-diabetic patients with renal involvement, the levels of protein (tyrosine) and polysaccharide remained within normal limits; in others they were distinctly elevated. In the complicated diabetic patients without renal disease (Group IIB) the proportion of protein to carbohydrate was fairly constant and similar to that in Groups I and IIA. However, in diabetic (Group IIC) and non-diabetic (Group III) patients with renal disease, a consistent relationship was not noted. The concentration of the protein (tyrosine) component was abnormally elevated in individual cases, but it remained rela-

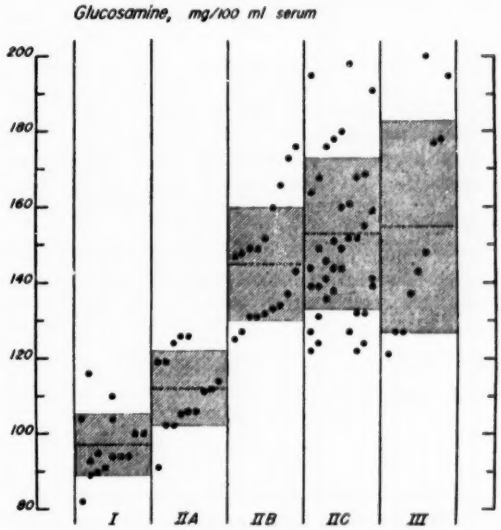

FIG. 2. SERUM GLUCOSAMINE CONTENT AMONG PATIENTS IN THE NON-DIABETIC CONTROL GROUP (I), UNCOMPLICATED DIABETIC PATIENTS (IIA), COMPLICATED DIABETIC PATIENTS WITHOUT RENAL INVOLVEMENT (IIB), AND COMPLICATED DIABETIC PATIENTS WITH RENAL INVOLVEMENT (IIC).

III denotes non-diabetic patients with renal disease. The dashed horizontal lines represent the mean in each group. The shaded area encloses the mean ± S.D.

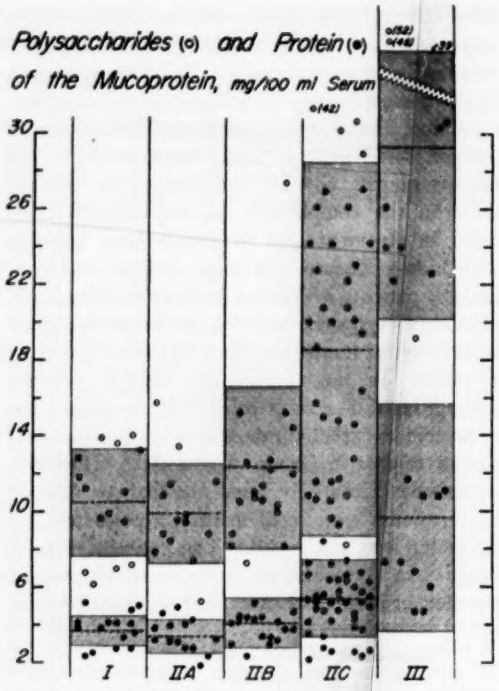

FIG. 3. THE NON-GLUCOSAMINE POLYSACCHARIDES (OPEN CIRCLES) AND PROTEIN (TYROSINE) CONTENT (SOLID CIRCLES) OF THE SERUM MUCOPROTEIN AMONG PATIENTS IN THE NON-DIABETIC CONTROL GROUP (I), UNCOMPLICATED DIABETIC PATIENTS (IIA), COMPLICATED DIABETIC PATIENTS WITHOUT RENAL INVOLVEMENT (IIB), AND COMPLICATED DIABETIC PATIENTS WITH RENAL DISEASE (IIC)

III designates non-diabetic patients with renal disease. The dashed horizontal lines represent the mean in each group. The mean ± S.D. is enclosed in the shaded area.

tively more constant than the carbohydrate levels which fluctuated widely.

DISCUSSION

Relatively little is known as yet of the chemical structure, function, and metabolism of the polysaccharides in the serum of normal individuals (18). The significance of modifications of their composition and concentration which have been noted in patients with a wide variety of diseases is correspondingly obscure. One factor common to the pathologic conditions in which such changes have been observed is tissue injury. Accordingly, elevation of the serum polysaccharide concentration is considered to be a non-specific index of destruction (7), or of proliferation and repair of tissue (5), perhaps reflecting the release into the circulation of substances derived from complex tissue carbohydrates. Experiments in which elevations of serum polysaccharides have been produced in animals by various types of tissue damage (19–22) lend some support to this concept.

Our results reveal that increased levels of certain polysaccharide substances in the sera of diabetic patients are associated with the existence of degenerative vascular complications, rather than with the primary metabolic defect in diabetes mellitus. Changes in the subintimal mucopolysaccharide ground substance have been implicated in the pathogenesis of arteriosclerosis (23, 24). One characteristic feature of the earliest sclerotic lesion in coronary arteries is a local increase in subendothelial mucoid ground substance (25). Our results may simply reflect such degenerative alterations widespread in blood vessels, and other structures. They do not exclude the possibility of a relationship of these increased circulating polysaccharides in such patients to the eventual development of specific glomerular and retinal capillary deposits of hyaline material. Because the diabetic patients in whom we observed abnormal serum polysaccharide concentrations already had well established degenerative vascular alterations, further data are required for clarification of these points. Serial observations are needed in the course of the development and progression of arteriosclerosis in diabetic as well as in non-diabetic individuals.

The changes in serum polysaccharide components in patients with kidney disease may represent the consequences of renal functional abnormalities, the effect of coexistent tissue alterations, or varying combinations of both these factors. In diabetic patients, the mean values for non-glucosamine polysaccharides of the serum protein, and for the serum glycosamine are elevated to comparable degree, and irrespective of the existence of abnormalities of renal function as evaluated by conventional clinical and laboratory methods. Combinations of extensive systemic vascular disease and renal dysfunction may account for the extreme increases in the values observed in individual patients. This possibility is further suggested by a progressive elevation of

polysaccharide substances which we have found in the sera of a few patients with diabetic glomerulosclerosis, concomitant with increasing proteinuria, following the administration of corticotropin (26).

In none of the groups did we note a consistent interrelationship of the concentrations of the serum polysaccharide components measured. Current knowledge of the structure and quantitative relationships of these substances in normal serum is insufficient to permit evaluation of this observation. It could merely be a reflection of the limitations of the methods used for their estimation. However, the fact that the polysaccharides in serum appear to be combined with protein, raises the question of the importance of changes in serum protein patterns to alterations of polysaccharide concentrations. It is known that the glycoproteins of normal serum are not homogeneous substances (18). The various fractions of normal serum protein bind different amounts of polysaccharides. Serum protein patterns may be altered differentially in various diseases, and the distribution of carbohydrates among the serum protein fractions may vary not only from that in normal serum, but also differentially with the disease (27). The sera of patients with diabetic glomerulosclerosis, analyzed by electrophoresis, contain increased concentrations of alpha-2 globulin and normal amounts of alpha-1 globulin (28). The elevated levels of protein-bound polysaccharides in the sera of our complicated diabetic patients with proven diabetic glomerulosclerosis would appear to be in keeping with the fact that, of the protein fractions in normal serum, alpha-2 globulin contains the highest percentage of polysaccharide (7). The normal amounts of serum mucoprotein in most of our diabetic patients correspond with current evidence that mucoprotein of the serum is a component of alpha-1 globulin (29). However, in patients with cancer and far advanced tuberculosis, actual values for serum polysaccharides could not be correlated with values calculated from concomitant increases in alpha-2 globulin measured by electrophoresis, and the polysaccharide content of protein fractions isolated from normal serum (30). Moreover, despite the fact that electrophoretic patterns of their sera are normal or characterized by a slight increase in beta globulins (28), hypertensive diabetic patients who do not have diabetic glomerulosclerosis, and arteriosclerotic diabetic patients without hypertension, have elevations of their serum polysaccharides of a type and degree comparable to that in patients with a fully developed Kimmelstiel-Wilson's syndrome. Without additional data, it cannot be stated whether the alterations in serum polysaccharides of diabetic patients with degenerative vascular disease reflect changes in the serum protein pattern, in the amounts of carbohydrate in normal protein components, or the appearance of abnormal circulating glycoproteins. In this regard, the variability of the carbohydrate to protein (tyrosine) ratio of the mucoprotein in some diabetic and non-diabetic patients with renal disease, as opposed to its relative constancy in the other groups, is of interest.

We observed a lack of correlation between the levels of the blood sugar and those of the various polysaccharide components which were measured. Our results thus fail to confirm a previous report (4) that there is a remarkable parallelism between blood sugar levels and glucosamine levels. This discrepancy may reflect differences in methods used to estimate glucosamine.

SUMMARY

1. The concentrations of total non-glucosamine polysaccharides bound to serum protein, serum glucosamine, and the protein (tyrosine) and polysaccharide of serum mucoprotein are within normal limits in diabetic patients without clinically detectable degenerative vascular disease.

2. In diabetic patients with degenerative vascular disease the total polysaccharides bound to serum proteins, and glucosamine of the serum, are increased. The values in such patients are not significantly influenced by the presence of renal involvement. While the mean values remain approximately normal, variations of the carbohydrate to protein ratio of the mucoprotein are found among complicated diabetic patients with proteinuria, renal insufficiency and hypertension. In contrast, this ratio is normal and relatively constant in such patients without renal abnormalities.

3. A uniform relationship between blood sugar levels and the various serum polysaccharide substances measured is not found.

4. The significance of these observations is discussed.

ACKNOWLEDGMENTS

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STUDIES ON INTRAVENOUS WATER DIURESIS AND NICOTINE AND PITRESSIN ANTIDURESIS IN NORMAL SUBJECTS AND PATIENTS WITH LIVER DISEASE [1]

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The fluid retention of patients with liver disease, leading to edema and ascites, has generally been attributed to primary sodium retention with secondary, obligatory water retention. However, because of the impaired water diuresis following oral hydration and the increased amounts of urinary antidiuretic activity observed in such patients, several investigators (1–4) have postulated some primary change in mechanisms influencing the release, physiological effect or inactivation of the neurohypophyseal antidiuretic hormone.

White, Rubin and Leiter (5) observed that, on the average, the maximal urine flows and periods of time required to achieve peak diuresis, during infusions of 10 ml. of intravenous glucose in water per minute, were the same in patients with liver disease and subjects with normal liver function. Furthermore, intravenous administration of 0.57 mU of Pitressin per Kg., during such water diuresis, produced antidiuresis of equivalent degree and duration in both groups. They concluded that there was no change in the rate of inactivation of either endogenous antidiuretic hormone or this dose of exogenous antidiuretic substance in patients with liver disease. More recently, Nelson and Welt (6) have confirmed these observations on essentially similar doses of Pitressin, *i.e.*, 2.5–100 mU intravenously. However, they found that when much larger doses of Pitressin were given subcutaneously to cirrhotic patients, there was delayed recovery from antidiuresis. They suggested that slower absorption of the large dose was the cause of the more prolonged antidiuresis.

These observations, however, do not exclude the possibility that excessive production of endogenous posterior pituitary antidiuretic hormone (ADH) or inadequate inactivation of larger doses of Pitressin or ADH might contribute to the retention of water in cirrhotic patients. Furthermore, the biological and chemical identity of commercial Pitressin and human endogenous posterior pituitary ADH has not been conclusively established. Therefore, the present studies were undertaken, using nicotine salicylate which has been demonstrated to stimulate posterior pituitary discharge of antidiuretic hormone in man (7–9). Patients with liver disease and recurring ascites on low salt diets were given very large intravenous water loads until maximum diuresis had been reached. Then, the degree and duration of the antidiuresis produced by the endogenous ADH, released after the intravenous injection of nicotine salicylate, were compared with the antidiuresis effected by Pitressin in "physiologic" doses intravenously.

EXPERIMENTAL MATERIALS AND METHODS

The subjects were 15 patients with cirrhosis of the liver, 12 of whom had varying degrees of demonstrable ascites. Three normal young female subjects and one patient with Addison's disease, who was maintained on 4 mg. of desoxycorticosterone acetate (DCA) and 25 mg. of cortisone daily, were also studied. All the cirrhotics and one of the normal subjects were on a low (25 mEq.) sodium diet. Two of the cirrhotic patients were studied twice.

The recumbent subjects, in the post-absorptive state, were given 1 liter of water orally in the morning one hour before the procedure. Then, diuresis was established by the intravenous administration of 5 per cent glucose in water at a constant rate, varying in the different subjects from 15 to 20 ml. per minute. Urine collections were obtained at approximately 10 minute intervals with the aid of sterile water and air washouts through a multi-holed soft rubber catheter. Arterial blood was drawn periodically through an indwelling femoral arterial needle.

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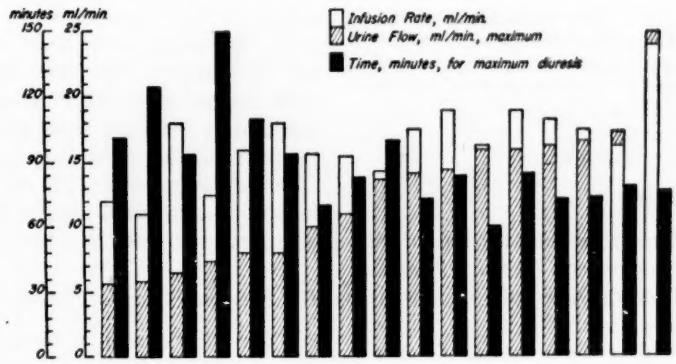

FIG. 1. MAXIMAL RESPONSE OF CIRRHOTIC PATIENTS TO INTRAVENOUS HYDRATION

When urine flows reached a constant maximal value in five of the cirrhotic patients, in the three normal subjects, and in the patient with Addison's disease, 1 to 3 mg. of nicotine salicylate (50 per cent alkaloid) were injected intravenously and urines collected until the flow had returned approximately to the previous control level. Then, a dose of Pitressin estimated to produce equivalent antidiuresis, 10 to 20 mU, was given intravenously and the resulting antidiuresis similarly measured. In several patients more than one dose of Pitressin was necessary to approximate the effect of the nicotine. To obviate the influence of any prolonged pharmacological effect, the order of injection of the two drugs was reversed in several instances. The procedure was repeated in two of the cirrhotic patients on subsequent days in order to test reproducibility of the observations.

Blood and urine samples were analyzed by the following methods: creatinine-like chromogen by Peter's modification of the Folin method (10), sodium and potassium with a lithium internal standard flame photometer,

TABLE I

Clinical status, creatinine clearance, and data on infusion diuresis of cirrhotic patients and subjects without liver disease

Patient	Age	Diagnosis	Degree of ascites	Maximal diuresis*			Creatinine clearance
				Infusion rate	Urine flow	Time to achieve	
				ml./min.	*ml./min.*	*min.*	*ml./min.*
R. Z.	40	Cirrhosis	4+	19.0	15.9	85	
R. W.	49	Cirrhosis	4+	18.0	6.50	94	31.4
R. C.	38	Cirrhosis	4+	18.7	8.00	97	
R. C.	38	Cirrhosis	4+	16.0	8.00	110	56
H. W.	48	Cirrhosis	3+	15.5	9.60	70	
M. B.	53	Cirrhosis	3+	16.5	10.9	83	84
J. V.	53	Cirrhosis	3+	19.0	14.4	84	102
T. Mc.	45	Cirrhosis	3+	12.0	5.58	101	30
J. V.	53	Cirrhosis	2+	17.4	16.4	74	85
B. F.	42	Cirrhosis	2+	17.5	14.2	73	101
B. F.	42	Cirrhosis	2+	18.2	15.3	73	104
M. M.	40	Cirrhosis	2+	16.6	19.0	79	
D. C.	28	Cirrhosis	1+	12.5	7.25	150	
R. F.	45	Hemochromatosis	1+	16.1	16.9	60	
A. C.	45	Cirrhosis	0	24.2	26.0	77	
W. M.	64	Cirrhosis	0	14.2	13.6	102	
O. S.	75	Cirrhosis	0	11.0	5.88	125	
F. E.	42	Addison's Dis.	0	17.2	12.6	60	72
M. S.	40	Normal	0	18.6	20.2	67	84
E. S.	23	Normal	0	17.7	14.1	85	81
M. J.	29	Normal	0	17.5	14.3	85	76

* During initial glucose infusion prior to administration of any antidiuretic materials.

chloride by the Van Slyke-Hiller modification of Sendroy's iodometric method (11), and glucose by an application of Somogyi's blood sugar method (12). Blood eosinophile counts were measured by a modification of Randolph's method (13).

RESULTS

I. Water Excretion

Figure 1 graphically presents the rate of infusion in each cirrhotic patient, the urine flows during peak diuresis and the time required to achieve maximal urine flow.

The ability of 10 of these patients to excrete large quantities of water during water loading is quite evident. At times the urine flows closely approximated, or even exceeded, the infusion rate. The maximal urine flow in five cirrhotic patients was less than 8 ml. per minute, despite much greater infusion rates. Of this group, one patient had known renal disease, three were accumulating fluid rapidly and had considerable ascites, and one was highly apprehensive and emotionally disturbed during the procedure. The time required to achieve maximal urine flows averaged 90 minutes.

Table I contains comparable data on the normal group and the Addisonian patient who was maintained on DCA and cortisone. These subjects achieved maximal urine flows comparable to the rate of infusion. The time required to achieve peak diuresis averaged 77 minutes, a value not significantly different from that of the 10 cirrhotics exhibiting good diuresis.

Because only traces of glycosuria were observed in occasional patients, the increased urine flows do not represent osmotic diureses.

II. Nicotine-Pitressin Antidiuresis

Figure 2 illustrates a typical procedure on a patient with cirrhosis and ascites. In the cirrhotics, the urine flow fell to a mean of 2.89 ml. per minute following nicotine and of 2.26 ml. per minute following Pitressin. The average duration of antidiuresis was 63 minutes after nicotine and 58 minutes after Pitressin.

In Table II are presented the results on the cirrhotic patients, the normal subjects, and the patient with Addison's disease. In the patients without liver disease, the duration of antidiuresis was 52 minutes following nicotine and 54 minutes following Pitressin. The intensity and duration of antidiuresis produced by the two drugs were essentially the same in all patients.

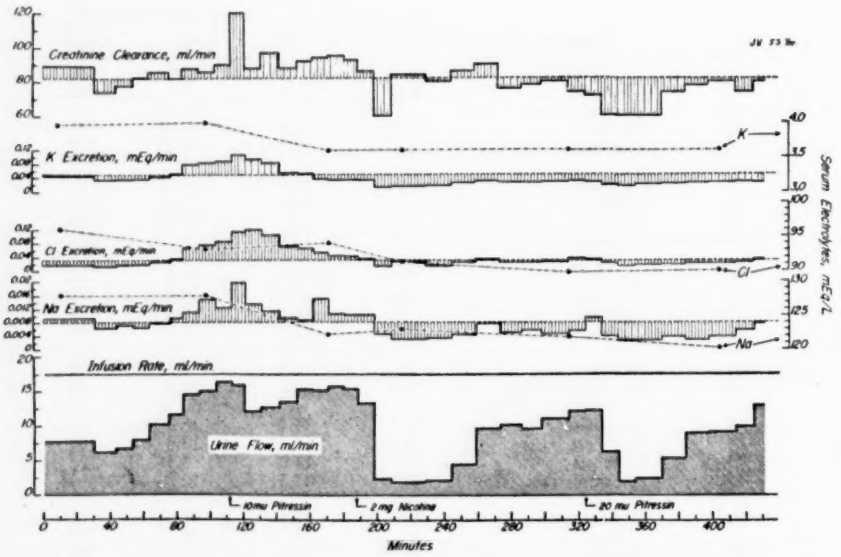

FIG. 2. COMPARISON OF PITRESSIN AND NICOTINE-INDUCED ANTIDIURESIS IN INTRAVENOUSLY HYDRATED CIRRHOTIC PATIENT ON LOW SALT DIET

TABLE II

Comparison of nicotine and Pitressin anti-diuresis in cirrhotic patients and subjects without liver disease

Patient	Diagnosis	Smoking	Drug	Dose	Water diuresis Max. urine fl.*	Antidiuresis Minimal U.F.†	Antidiuresis Duration
					ml./min.	*ml./min.*	*min.*
H. W.	Cirrhosis	0	Nicotine	2 mg.	8.50	3.70	50
			Pitressin	10 mU	7.50	1.82	44
M. B.	Cirrhosis	0	Nicotine	2 mg.	10.9	1.20	62
			Pitressin	10 mU	10.7	2.00	44
J. V.	Cirrhosis	0	Nicotine	2 mg.	20.0	2.38	78
			Pitressin	20 mU	14.4	2.71	58
J. V.	Cirrhosis	0	Nicotine	2 mg.	15.7	2.00	74
			Pitressin	20 mU	12.2	2.00	60
B. F.	Cirrhosis	0	Nicotine	3 mg.	15.4	2.40	76
			Pitressin	10 mU	15.3	2.60	63
B. F.	Cirrhosis	0	Nicotine	—	15.4	—	—
			Pitressin	10 mU	14.2	2.60	60
M. M.	Cirrhosis	+	Nicotine	3 mg.	19.0	5.70	38
			Pitressin	20 mU	17.2	2.00	79
F. E.	Addison's Dis.	0	Nicotine	2 mg.	12.6	6.80	44
			Pitressin	5 mU	17.9	6.00	65
M. S.	Normal	0	Nicotine	2 mg.	25.0	9.20	37
			Pitressin	10 mU	20.2	7.75	35
E. S.	Normal	+	Nicotine	3 mg.	13.9	6.56	60
			Pitressin	10 mU	14.1	1.75	60
M. J.	Normal	+	Nicotine	3 mg.	14.4	4.90	67
			Pitressin	10 mU	14.3	1.66	60

* Just prior to giving Pitressin or nicotine.
† U. F. = Urine flow.

III. Urinary Excretion annd Serum Concentrations of Electrolytes

Little change was noted in urinary electrolyte excretion during the glucose in water infusions, despite the large changes in water excretion. An increase in urinary sodium, chloride and potassium excretions was noted during the early periods of hydration (cf. Figure 2) in all the patients except the three seriously ill cirrhotic patients, who exhibited little diuresis on water loading. This may represent normal diurnal variation in electrolyte excretion, since the increase occurred at the time of expected matutinal rise (14). The other three, sicker patients may have had the reported reversal of the typical diurnal tide (15).

DISCUSSION

The present data on the time for attainment of peak diuresis during intravenous hydration and the duration of antidiuresis produced by "physiologic" doses of Pitressin in patients with and without liver disease agree with previously reported observations (5, 6). Despite the somewhat smaller dosage of Pitressin used in this study, the duration of the antidiuretic effect was the same in both groups. Thus, in these cirrhotic patients there was no evidence of either increased renal tubular sensitivity to, or of decreased inactivation of, exogenous antidiuretic hormone at this dosage.

Since the time necessary to achieve maximal diuresis was similar in the cirrhotic and normal subjects, inactivation of circulating endogenous ADH apparently occurred at the same rate in both groups, once posterior pituitary discharge had been inhibited via osmoreceptors and possibly volume receptors[4] (16). Such complete inhibition

[4] The failure to observe greater increase in electrolyte excretion during maximal diuresis than would be

suggests that the mechanisms regulating ADH release, if contributing at all to the fluid retention in these patients, still respond to these excessive water loads. However, in cirrhotic patients, as in cardiacs (17), acute antidiuretic mechanisms may be activated, under certain circumstances, which lead to water retention *per se*, despite expansion of extracellular fluid and hyponatremia. It remains to be established whether or not such sustained production of antidiuretic hormone is the responsible mechanism in these cases.

That nicotine acts to stimulate posterior pituitary secretion of antidiuretic hormone is well known. Burn, Truelove and Burn in 1945 (7) noted that 0.1 mg. of nicotine suppressed the urine flow in the hydrated rat but not in hypophysectomized rats maintained on anterior pituitary extract, and that in human subjects cigarette smoking produced similar inhibition of water diuresis. Walker subsequently also reported that cigarette smoking and intravenous injections of nicotine stimulate ADH discharge in normal man (8). Chalmers and Lewis (18) and Cates and Garrod (19) have recently suggested that the failure of nicotine to affect urine flow in a hydrated patient may be a positive test for diabetes insipidus. The equivalent antidiuresis observed in both our cirrhotic patients and normal subjects following nicotine salicylate and Pitressin suggests that endogenous ADH and aqueous Pitressin are physiologically similar in this respect. Moreover, it indicates that under the conditions of these studies the posterior pituitary of the cirrhotic patients responded physiologically but not excessively to a standard pharmacological stimulus.

The transient and inconstant changes in endogenous creatinine clearance occurring in the cirrhotic and normal subjects after Pitressin and nicotine probably reflect errors in collection due to changes in urine flow. Such an interpretation is consistent with the established view that the antidiuresis is largely independent of changes in filtration.

Three cirrhotic patients (R. C., R. W., T. Mc. in Table I) had much lower endogenous creatinine clearances than the patients who exhibited good diuresis. These three patients were extremely ill and were accumulating ascitic fluid rapidly. Therefore, their poor diuretic response may not be related specifically to antidiuretic hormone, but may be a consequence of impaired renal hemodynamics, in part due to mechanical compression of the renal veins and ureters by the large amount of fluid accumulated in the abdomen. In addition, if fluid were rapidly transferred to the peritoneal cavity during the glucose infusions, little expansion of the effective circulating volume would occur. Consequently, inhibition of volume receptors, if these were overactive, might not have been achieved.

Since blood eosinophil counts, taken at intervals for several hours after nicotine and Pitressin administration, did not change significantly in either group of patients, discharge of adrenal cortical hormone probably did not occur during these procedures. In this connection, it is interesting that the treated patient with Addison's disease also gave a typical, normal response to both Pitressin and nicotine.

The factors initiating salt and water retention in patients with cirrhosis of the liver are still obscure. Whenever the delicate balance of forces regulating electrolyte and water excretion in such patients is upset, there may be intensification of a number of mechanisms promoting fluid retention. When this occurs in patients on very restricted salt intakes, primary retention of water without sodium may result, leading to weight gain and hyponatremia, similar to that we have observed in edematous cardiacs (20). Although it is possible that sustained production of antidiuretic hormone, in the face of dilution of the body fluids, may be responsible for this water retention (21), at present, determination of the activating mechanisms or the precise role of the neurohypophysis in this process is difficult.

The present data contain no evidence of decreased inactivation, persistent hypersecretion, or increased renal tubular effects of endogenous ADH in our edematous cirrhotics. However, the acute occurrence of water retention in cirrhotics would suggest that a similar process may contribute to their usual, more chronic, fluid retention. An accurate method for measuring circulating ADH at physiologically effective blood levels is required to settle this problem.

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expected on the basis of diurnal variation suggests that volume receptor inhibition may not have been of significance.

SUMMARY AND CONCLUSIONS

1) Seven of 11 patients with liver disease and varying degrees of ascites achieved a maximal urine flow of 9.6 to 19.0 ml. per minute, in response to the intravenous infusion of 5 per cent glucose solution at rates of 12.0 to 19.0 ml. per minute. Creatinine clearances were reduced to 31.4 to 56 ml. per minute in three of the four patients with smaller peak diuresis, in whom these measurements were made.

2) The intravenous injections of 1 to 3 mg. of nicotine salicylate in intravenously hydrated cirrhotic and normal subjects produced an antidiuretic response equivalent in degree and duration to that resulting from the intravenous injection of 5 to 20 mU of Pitressin in the same subjects.

3) Ten cirrhotic patients with good diuresis required an average of 79 minutes to achieve maximal diuresis. This normal value points against hypersecretion or impaired inactivation of ADH in these patients, eight of whom had ascites.

4) The essentially similar antidiuretic response of cirrhotic patients and normal subjects to intravenous nicotine salicylate and Pitressin (endogenous and exogenous ADH) seems to exclude increased renal tubular sensitivity to ADH in cirrhosis of the liver. The equivalent duration of this antidiuresis in cirrhotic and normal subjects is evidence against abnormal inactivation of ADH in the former.

5) These studies confirm the absence of significant changes in endogenous creatinine clearance and in urinary electrolyte excretion after injection of physiological amounts of Pitressin or endogenous ADH in the water-loaded subject.

6) The failure to achieve adequate water diuresis in certain cirrhotic patients may be attributed in part to abnormal renal hemodynamics.

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INTERPRETATION OF THE RAPID INTRAVENOUS GLUCOSE TOLERANCE TEST IN NORMAL INDIVIDUALS AND IN MILD DIABETES MELLITUS [1]

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The glucose tolerance test is an important diagnostic procedure in metabolic diseases. As the test is currently employed, glucose is administered either orally or intravenously and there is *no uniform* interpretation of the results.

A study of the rapid glucose tolerance test is found in a review by Tunbridge and Allibone (1). The present study was undertaken to test the validity of the concept that the rapid intravenous glucose tolerance test might properly and readily be interpreted by following the rate of disappearance of glucose from the blood. It was our hope also to evolve a simple test for routine use. The uniformity of the single injection method was studied as a means of separating the normal from the known mild diabetic patient.

MATERIAL AND METHODS

Twenty-five grams of glucose as a 30 per cent solution in distilled water were administered intravenously within four minutes. A fasting blood specimen was obtained, and then at four minutes following the glucose administration and subsequently every eight minutes for 72 minutes. Blood was obtained from an ear lobe that was kept moist with a heparin sponge. Determination of blood glucose was done by the Horvath and Knehr modification (2) of the Folin-Malmros micromethod (3).

The control group consisted of 70 healthy men ranging in age from 25 to 50 years with no family history of diabetes mellitus. The test was repeated with 25 grams of glucose at intervals of from one to three months in 20 subjects, and with 35 grams of glucose in 13 who had been on an adequate carbohydrate intake. The abnormal group consisted of 26 patients with known mild diabetes mellitus controlled by diet alone (the majority had normal fasting blood sugars) and 13 patients with severe diabetes mellitus. Diabetes mellitus had been diagnosed in these patients by clinical evaluation and the 100 grams standard oral glucose tolerance test.

RESULTS

The blood glucose values of the 25 grams rapid glucose tolerance test, expressed as mgm. per cent glucose excess,[3] in 70 normal men with no family history of diabetes mellitus are given in Table I. Similar blood (mgm. per cent) glucose excess values were found in 20 normal men who had a repeat 25 grams test one to three months later

[3] Milligram per cent glucose excess is defined as that amount of glucose in excess of the fasting blood glucose in mgm. per cent.

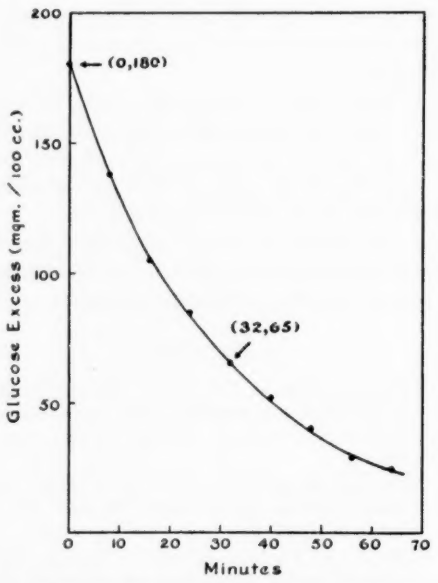

FIG. 1. GLUCOSE DISAPPEARANCE PLOTTED ON COORDINATE GRAPH PAPER—PATIENT W. B.

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and who had been on an adequate carbohydrate intake (Table II). A comparison of the 25 grams to the 35 grams test showed different mgm. per cent glucose excess values at determined times in 13 normal men who had been on an adequate carbohydrate intake (Table IV). In 39 patients with diabetes mellitus (26 were mild and 13 were severe diabetics) the 25 grams glucose tolerance test gave higher mgm. per cent glucose excess values (Table V).

The rate of disappearance of glucose was found to be apparently proportional to the concentration if the glucose excess in mgm. per 100 cc. is plotted against time using the four minute glucose value after injection as that value at time zero (Figure 1). This is best represented by an equation of a monomolecular reaction:

$$C(t) = C(o)\, e^{-kt} \qquad (1)$$

where

$C(t)$ = the glucose excess in mgm. per 100 cc. at any time, t,

$C(o)$ = the glucose excess in mgm. per 100 cc. at time zero,

and

k = the disappearance rate of glucose from the blood.

TABLE I

The rapid intravenous glucose tolerance (25 grams) and its rate of removal in normal men

Patient	Fasting glucose	Glucose excess (mgm.%) 0	8	16	24	32 (time in minutes)	40	48	56	Rate of removal (%/min.)
C. B.	78	182	140	100	80	57	47	35	17	3.44
P. D.	88	207	142	109	77	65	38	27	12	4.30
R. P.	104	140	91	66	44	31	20	1	1	4.81
S. B.	105	151	95	80	60	47	33	10	1	3.75
G. S.	115	145	108	73	45	34	25	−6	−6	4.60
P. W.	93	215	155	107	82	39	40	30	−10	3.98
R. G.	93	205	150	108	71	50	28	15	−6	4.53
J. D.	93	169	122	94	65	50	41	34	15	3.51
L. I.	95	160	121	83	58	40	40	23	6	4.21
P. K.	90	225	173	133	110	85	77	55	30	3.05
W. B.	122	153	106	76	56	41	28	5	1	4.14
A. K.	115	195	141	100	73		30	10	−13	4.53
M. C.	88	199	141	104	70	52	34	12	−8	4.37
D. C.	91	211	157	119	105	103	72	57	41	3.00
W. B.	123	173	120	85	65	45	35	23	7	4.06
C. C.	88	195	134	100	72	52	28	23	16	4.06
W. K. N.	84	241	184	142	104	89	76	50	42	3.12
V. C.	83	183	124	95	67	51	42	29	19	3.90
W. B.	85	177	138	105	85	65	52	40	29	3.20
R. C.	97	183	141	109	82	65	53	41	26	3.20
M. W.	85	157	112	82	67	56	48	36	18	3.30
W. W.	98	192	139	107	87	64	50	25	9	3.44
G. T.	93	179	140	101	81	59	50	35	19	3.51
J. J.	95	182	152	98	81	70	50	38	17	3.08
K. H.	128	190	132	124	89	57	46	34	25	4.06
E. M.	100	140	116	86	69	50	38	30	8	3.43
H. O.	90	175	126	100	78	56	48	36	20	3.35
E. R.	80	191	165	118	95	75	58	48	36	3.32
L. E.	92	188	143	105	85	69	52	36	29	3.20
C. L.	78	197	132	114	73	55	43	28	22	3.90
A. S.	105	160	113	81	67	51	36	32	22	3.49
F. Mc.	92	183	125	91	62	41	26	18	8	4.84
C. A.	83	204	141	94	75	59	34	28	10	4.16
B. H.	81	167	115	85	55	29	29	21	−3	4.45
E. S.	95	178	140	107	87	68	43	42	23	3.04
S. R.	88	150	111	92	72	59	46	13	25	3.12
H. H.	95	165	118	95	70	51	37	29	11	3.67
K. N.	97	158	131	106	86	73	49	36	28	3.04
J. S.	87	164	135	110	82	61	42	35	23	3.82
B. D.	97	167	116	88	71	52	28	28	1	3.95

TABLE I—*Continued*

Patient	Fasting glucose	Glucose excess (mgm.%) 0	8	16	24	32	40	48	56 (time in minutes)	Rate of removal (%/min.)
R. F.	87	206	143	101	85	54	40	28	10	4.30
T. R.	82	188	133	96	68	44	35	20	14	4.10
R. C. H.	87	185	134	95	74	48	35	17	5	4.53
F. R.	91	230	150	124	114	84	67	57	36	3.35
E. E.	110	173	113	80	50	35	25	−3	−5	4.84
D. R. U.	102	161		100	78	56	38	28	13	3.98
V. K.	102	165	123	103	76	62	45	33	24	3.36
S. L.	93	137	113	98	72	55	42	37	25	3.36
H.	98	177	120	92	76	53	41	27	15	3.84
R. A. J.	82	156	114	88	74	51	40	30	13	3.46
G. F. E.	95	138	99	77	57	43	38	27	21	3.38
E. G.	85	187	143	97	63	43	30	−1	−12	4.62
M. K.	95	153	119	100	82	71	57	38	31	3.01
A. Z.	78	196	142	117	90	77	53	46	27	3.30
I. P.	84	180	144	112	82	55	28	7	−3	3.74
W. G.	88	169	123	97	72	53	50	30	17	3.56
W. M.	98	174	133	118	102	74	57	46	34	3.08
W. J.	87	168	130	101	76	58	35	28	13	3.64
E. G.	86	175	133	107	94	76	55	45	34	3.01
S. D.	97	128	107	76	65	53	39	30	26	3.20
N. H.	102	159	118	96	84	72	64	46	34	3.00
G. D.	98	182	138	113	85	62	40	37	12	3.36
W. L.	102	151	108	86	54	56	51	31	24	3.34
A. B.	97	138	109	83	63	52	39	30	21	3.44
F. L. T.	92	156	114	76	46	37	0	8	−18	4.62
H. H.	102	159	118	96	84	72	64	46	34	3.00
B. K.	75	170	129	100	79	58	43	19	16	3.55
L. A. S.	92	173	126	86	58	40	0	1	−10	4.62
M. P.	98	149	110	87	70	52	41	23	20	3.30
A. S.	122	116	80	69	53	42	30	14	11	3.22

A simple expression for finding the disappearance rate of glucose is to let $C(t) = \frac{1}{2} C(o)$, then on substituting in eq. (*1*)

$$\tfrac{1}{2} C(o) = C(o)\, e^{-kt}$$

or

$$k = \frac{\log_e 2}{t} = \frac{0.693}{t} \qquad (2)$$

ANALYTIC AND GRAPHIC ANALYSIS

As it is well appreciated in the application of mathematics to practical problems, one deals with inaccurate data. All measurements entering into the calculation involve errors of some magnitude and many of the numerical values used are rounded off. If one plots the observed mgm. per cent of glucose during the rapid glucose tolerance test against time, a parabola is formed. However, assuming that the fasting blood glucose is a constant baseline during the rapid glucose tolerance test, then by plotting $\log_e$ (mgm. per cent glucose excess) against time one will get a linear rela-

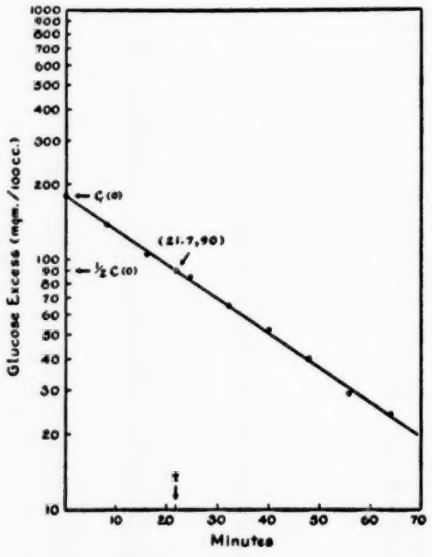

FIG. 2. GLUCOSE DISAPPEARANCE OF FIG. 1 PLOTTED ON SEMILOGARITHMIC PAPER—PATIENT W. B.

tionship (eq. [1]). By fermentation we found that the non-glucose reducing substances during the rapid glucose tolerance test remained relatively constant (the greatest difference being 5 mgm. per cent) in seven normal individuals and six patients with diabetes mellitus. It should be emphasized that any errors inherent in the method, technical or personal, may, at times, be magnified so as not to give a good fit on plotting $\log_e$ (mgm. per cent glucose excess) against time. From this graph one can readily find k (the disappearance rate of glucose from the blood) by eq. (2). This is graphically illustrated by plotting glucose excess on the ordinate of semilogarithmic paper against time on the abscissa (Figure 2), where k is the number of multiples of 2.30 minutes corresponding to each power of 10 through which the glucose excess changes. An analytical and graphical sample is given in Table III.

a. Analytical method

The graph of the relation between the blood mgm. per cent glucose excess and time is shown in Figure 1. Substituting into eq. (1), one readily

TABLE II

The rapid intravenous glucose tolerance (25 grams) and a repeated (25 grams) test with its rate of removal in normal men

Patient		Fasting glucose	Glucose excess (mgm.%) 0	8	16	24	32 (time in minutes)	40	48	56	Rate of removal (%/min.)
R. C.	a.*	83	183	124	95	67	51	42	35	19	3.90
	b.	91	177	130	82	57	44	35	21	14	4.06
W. B.	a.	85	177	138	105	85	65	52	40	29	3.20
	b.	80	165	123	103	84	67	50	38	23	3.20
R. C.	a.	97	183	141	109	82	65	53	41	26	3.20
	b.	105	184	147	104	79	68	48	38	15	3.28
M. W.	a.	85	157	112	82	67	56	48	36	18	3.20
	b.	117	118	80	70	55	43	38	23	17	3.36
G. T.	a.	93	179	140	101	81	59	50	35	19	3.59
	b.	80	230	160	128	93	72	54	33	29	3.60
Dr. J.	a.	95	182	152	98	81	70	50	38	17	3.08
	b.	80	176	148	107	80	70	55	47	23	2.97
K. H.	a.	128	190	132	124	89	57	46	34	25	3.98
	b.	128	172	120	87	59	46	16	8	8	4.06
E. R.	a.	80	191	165	118	95	75	58	48	36	3.24
	b.	100	187	143	102	86	62	48	29	21	3.35
L. E.	a.	92	188	143	105	85	69	52	36	29	3.20
	b.	90	216	160	135	105	83	66	53	38	3.20
F. Mc.	a.	92	183	125	91	62	41	26	18	8	4.84
	b.	87	203	147	103	61	55	30	15	6	4.92
C. A.	a.	83	204	141	94	75	59	34	28	10	4.16
	b.	83	207	147	108	72	65	36	19	7	4.21
H. H.	a.	95	165	118	95	70	51	37	29	11	3.67
	b.	89	180	137	107	76	55	47	38	11	3.67
H. N.	a.	97	158	131	106	86	73	49	36	28	3.04
	b.	96	200	150	109	95	82	71	64	55	3.00
B. D.	a.	97	167	116	88	71	52	28	28	1	3.95
	b.	102	140	93	74	48	29	27	19	4	3.98
F. R.	a.	91	230	150	124	114	84	67	57	36	3.35
	b.	85	160	139	103	81	58	45	36	28	3.43
G. F. E.	a.	95	138	99	77	57	43	38	27	21	3.38
	b.	83	147	105	83	57	45	43	26	20	3.41
A. Z.	a.	78	196	142	117	90	77	53	46	27	3.30
	b.	83	164	125	95	75	59	48	42	28	3.31
E. G.	a.	85	187	143	97	63	33	9	−1	−12	4.62
	b.	93	167	119	80	46	36	−5	−18	−20	4.62
R. C. H.	a.	87	185	134	95	74	48	35	17	5	4.53
	b.	92	138	102	67	45	33	14	3	−11	4.62
W. W.	a.	98	192	139	107	87	64	50	25	9	3.36
	b.	90	186	135	115	95	74	53	40	23	3.36

* a. 25 grams of Glucose.
b. 25 grams of Glucose.

TABLE III

Observed blood glucose values of W. B., with the four minute value set at time zero

Time (minutes)	Fasting	4	12	20	28	36	44	52	60	68
Observed glucose (mgm.%)	85	265	223	190	170	150	137	125	114	109
Time (4 minutes at time 0)		0	8	16	24	32	40	48	56	64
Glucose excess (mgm.%)		180	138	105	85	65	52	40	29	24

finds the k, the disappearance rate of glucose from the blood for two points.

$$k = \frac{2.3 \log_{10} 180/65}{32-0} = 0.0319$$

or

$$k = 3.19 \text{ per cent per minute.}$$

b. Graphic method

The semilogarithmic graph of the relation between the blood glucose excess and time is illustrated in Figure 2.

By using the relationship shown in eq. (*2*) where C(t) equals $\frac{1}{2}$ C(o), to find t from Figure 2 we see that $\frac{1}{2}$ C(o) equals 90 mgm. per 100 cc. with a corresponding t equal to 21.7 minutes. Substituting into eq. (*2*), one finds the average disappearance rate of glucose.

$$k = \frac{0.693}{21.7} = 0.0319$$

or

$$k = 3.19 \text{ per cent per minute.}$$

The skill required for drawing an accurate freehand curve is not required with the graphic method and the average k for all points is more readily and easily obtained.

It appears that the disappearance rate of glucose from the blood is apparently the resultant of a number of simultaneous processes that best fit a simple unimolecular equation. The disappearance rate of glucose in 70 normal men with no family history of diabetes mellitus who received 25 grams of glucose intravenously was found to range from

TABLE IV

The removal rate on comparing the 25 grams and 35 grams rapid intravenous glucose tolerance in normal men

Patient		Fasting glucose	Glucose excess (mgm.%) (time in minutes) 0	8	16	24	32	40	48	56	64	72	Rate of removal (%/min.)
R. C.	a.*	105	184	149	103	79	68	48	38	15	15	10	3.28
	b.	96	249	200	143	112	87	74	35	15	−6	−1	3.28
G. T.	a.	93	179	140	101	81	59	50	35	19	8	0	3.59
	b.	84	311	228	191	140	109	89	69	49	35	14	3.51
Dr. J.	a.	80	176	148	107	80	70	55	47	23	6	0	2.97
	b.	92	256	192	171	143	118	95	75	75	46	34	2.97
L. E.	a.	92	188	143	105	85	69	52	36	29	16	4	3.20
	b.	96	302	209	161	126	94	79	49	32	16	−5	3.20
F. Mc.	a.	87	203	147	103	61	55	30	15	6	6	−2	4.92
	b.	96	214	166	114	69	46	47	24	16	10	2	4.71
E. S.	a.	95	178	140	107	87	68	43	42	23	17	0	3.04
	b.	90	238	188	172	140	120	98	84	73	55	34	3.12
R. S.	a.	88	150	111	92	72	59	46	13	25	17	7	3.12
	b.	88	204	165	130	120	92	72	55	37	32	12	3.05
H. H.	a.	89	180	137	107	76	55	47	38	11	3	−4	3.67
	b.	77	227	184	144	109	85	56	45	30	19	8	3.67
K. N.	a.	96	200	150	109	95	82	71	64	55	41	32	3.00
	b.	87	198	170	138	124	105	83	61	49	38	38	3.00
J. S.	a.	87	164	135	110	82	61	42	35	23	8	3	3.82
	b.	98	197	148	115	91	59	46	29	18	17	9	3.90
B. D.	a.	102	140	93	74	48	29	14	19	4	−4	−12	3.98
	b.	105	245	177	149	120	100	74	48	35	20	11	3.98
R. F.	a.	87	206	143	101	85	54	40	28	10	4	−10	4.30
	b.	100	230	150	107	73	46	22	22	−10	−27	−22	4.60
R. C. H.	a.	87	185	134	95	74	48	35	17	5	−4	−5	4.21
	b.	91	230	147	109	87	71	54	25	13	0	0	4.21

* a. 25 grams of Glucose.
b. 35 grams of Glucose.

TABLE V

The rapid intravenous glucose (25 grams) tolerance test in diabetes mellitus

Patient	Fasting	Glucose excess (mgm.%) 0	8	16	24	32	40 (time in minutes)	48	56	64	72	Rate of removal (%/min.)
						Very mild diabetes mellitus						
O. D.	105	187	168	142	123	105	90	71	63	38	38	2.03
W.	94	149	134	109	100	88	81	66	48	42	19	1.64
E. J.	95	170	138	119	108	90	83	73	53	48	37	1.79
R. S.	147	106	99	89	78	71	61	60	57	43	48	1.21
L. L.	146	174	136	110	102	78	68	48	44	38	30	2.46
A. R.	160	236	172	156	130	116	100	94	72	64	70	1.83
M.	115	153	138	105	98	85	79	79	46	28	35	2.10
O. T.	98	202	157	139	121	112	102	65	58	45	47	2.27
D. H.	105	130	105	90	75	67	60	52	45	38	37	1.83
W. G.	97	213	155	128	114	98	93	89	54	40	34	2.42
M. B.	94	179	134	117	100	88	73	66	52	42	31	2.22
O.	80	210	153	138	118	105	95	77	56	35	31	2.18
B.	94	153	124	111	100	92	78	71	56	43	31	1.79
A. P.	102	134	113	93	83	78	65	58	45	28	23	1.87
W. S.	135	135	110	93	68	65	59	51	40	20	30	2.07
C. F.	95	175	132	121	113	85	71	63	45	33	16	2.42
W. W.	115	125	116	102	87	75	68	64	62	56	56	1.26
A. K.	158	122	100	75	64	52	43	31	28	20	19	2.19
E. C.	145	154	125	99	85	72	60	49	40	35	27	2.32
W. W.	143	119	106	101	90	79	81	72	71	65	56	0.93
J. E.	111	143	131	119	107	99	90	83	81	72	66	1.09
J. L.	113	142	130	112	97	82	76	65	55	45	36	1.77
R. S.	104	148	124	103	84	76	60	47	44	36	29	2.35
B. O.	134	131	120	114	99	99	88	83	76	69	62	0.99
E. F.	104	149	143	126	126	109	110	99	91	74	78	0.93
F. S.	89	125	109	99	83	78	73	67	61	55	53	1.23
						Severe diabetes mellitus						
J. U.	206	210	160	124	116	110	106	84	70	62	50	1.64
G. E.	322	152	116	114	102	104	78	76	74	66	60	1.13
M. C.	136	200	154	124	124	104	104	104	82	82	82	1.09
E. J.	224	196	136	108	96	84	80	76	50	48	46	1.87
B. P.	155	161	152	146	132	122	108	109	102	102	87	0.85
A. N.	155	133	99	97	90	75	77	66	59	54	57	1.12
E. W.	198	65	73	70	67	64	62	65	65	62	64	0.23
F. D.	228	90	72	64	64	56	52	47	43	39	39	0.97
A. D.	192	112	100	92	81	76	69	63	61	58	56	1.06
W. H.	168	222	176	158	146	132	136	102	92	82	80	1.48
O. N.	192	64	62	58	57	61	63	61	57	57	55	0.15
A. B.	262	76	70	62	58	49	45	46	43	36	30	1.23
A. D.	168	110	110	100	92	85	79	75	74	66	52	0.85

3.00 to 4.84 per cent per minute (Table I). The test was repeated with 25 grams of glucose one to three months later on 20 normal subjects who had a dietary intake of at least 150 grams of carbohydrate daily. The disappearance rate of glucose (k) was constant for each subject (Table II), *i.e.*, the disappearance rate was reproducible.

In order to study the effect of a larger dose the test was repeated with 35 grams of glucose in 13 normal individuals who had been previously studied with the 25 grams dose and who had a dietary intake of at least 150 grams of carbohydrate daily (Table IV). It was found that the disappearance rate was unaltered by the larger dosage, *i.e.*, it was constant for the individual tested. Figure 3 illustrates the observed data of the two dosage tests in subject W. B. plotted on semilogarithmic graph paper. It is obvious that the slopes of the curves, and therefore the removal rates, are the same and are unaltered by this change in dosage. It should also be noted that an apparent oscillation of the curve is exaggerated with the 35 grams dose but

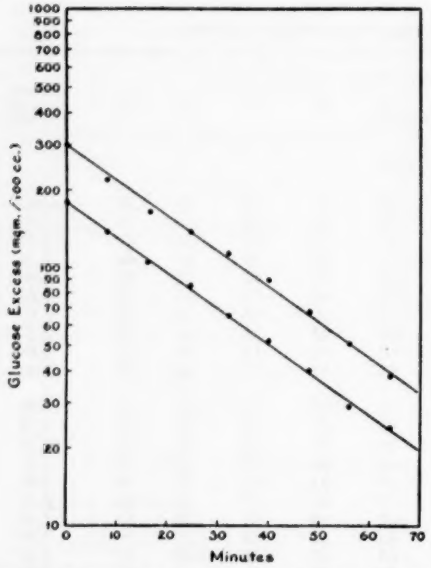

FIG. 3. COMPARISON OF THE GLUCOSE DISAPPEARANCE AFTER 25 AND 35 GRAM TEST OF GLUCOSE—PATIENT W. B.

that the values of the disappearance rate of glucose following dosages of 25 and 35 grams are the same. The apparent oscillations may be related to numerous simultaneous processes in the body.

A group of 26 patients with known mild diabetes controlled by diet (the majority had normal fasting blood sugar values) was similarly studied with the 25 grams test. The disappearance rate of glucose was found to range from 0.93 to 2.46 per cent per minute (Table V). This difference is obviously highly significant on comparison with the normal series. A distinct, sharp separation of the mild diabetic patients from the normal subjects is readily apparent. Also studied were 13 patients with severe diabetes who were found to have a lower range (0.23 to 1.64 per cent per minute) for the rate of removal of glucose (Table V).

The mean quantity of glucose lost in the urine during the test varied from 0.2 to 1.0 gram in 22 normal men, and from 0.7 to 2.9 grams in 19 mild and severe diabetics.

DISCUSSION

The first attempt to analyze the rapid glucose tolerance curve was by Jörgenson (4) (1926) and Ross (5) (1938) who measured the area under the observed curve to interpret their data. Fishberg (6) (1930) found that the rate of disappearance of foreign sugars injected into the blood stream obeyed a monomolecular reaction velocity equation. An excellent detailed treatment for determining the intravenous glucose tolerance equation was shown by Greville (7).

The fate of the intravenously administered glucose has not been definitely determined. Loss of glucose in the urine is not an important factor since only a small amount was found in the patients tested (0.2 to 2 grams). The disappearance of glucose from the blood merely by diffusion into the tissues is unlikely because: (*1*) the disappearance rate of glucose from the blood in mild diabetics and in normal individuals with a similar fasting blood sugar level showed a distinct difference, and (*2*) the disappearance rate did not vary with the dose of glucose. It may be suggested that changes in blood volume resulting from the injected glucose may produce an apparent loss from the blood stream. However, Pijoan and Gibson (8) using the Evans blue dye method found that there was no significant change in blood volume after the injection of 25 grams of glucose in 55 cc. of water.

The ferricyanide reaction as a method of determining blood glucose was introduced by Hagedorn and Jensen (9, 10). For routine purposes the disadvantage of this procedure was that it measured all *reducing* substances in the blood in addition to glucose. Measurement of the mgm. per cent *glucose excess* in blood, determined by the difference in reducing power before and after the injection of glucose, avoids consideration of the non-glucose-reducing substances and thus, for this purpose, the ferricyanide procedure becomes the method of choice.

The variation in the disappearance rate of glucose in normal individuals could not be correlated with the height, weight (*i.e.*, non-obese patients), age or dose administered. It was apparent that the disappearance rate was reproducible for the individual regardless of dose. An apparent oscillation of the plotted curves was noted with the 35 grams injection. By the rapid glucose tolerance test, one can obtain an index of the disappearance rate of glucose from the blood. With values of glucose excess below 30 mgm. per 100 cc. the plotted curve tends to become distorted.

The above interpretation has some advantages in that (*1*) the disappearance rate of glucose in normals is constant, reproducible, and independent of dosage for each individual, (*2*) there is a marked separation of the normal from known borderline diabetes mellitus, (*3*) a precise manner of interpreting glucose tolerance curves is possible, (*4*) a rapid glucose tolerance test may be completed within 60 minutes, and (*5*) the disappearance rate of glucose from the blood appears to fit best a simple expression (eq. [*1*]).

SUMMARY

A study has been made of the rapid intravenous glucose tolerance test by following the disappearance rate of glucose. It was found that the rate of removal in the normal individual maintained on an adequate carbohydrate diet remained constant on repeated tests and was independent of the dose administered for each subject.

By determining the removal rate, a sharp separation was found between the patients with known mild diabetes (*26*), severe diabetes (*13*), and the group of normal controls (*70*). A decreased rate of removal has been observed in other conditions, including obesity unrelated to a family history of diabetes, inflammatory disease, uremia, weight loss due to diet, and decompensated portal cirrhosis.

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THE EFFECT OF SACCHARIDES ON LEUKOCYTE MOVEMENT [1]

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INTRODUCTION

The movement of leukocytes has been associated with the presence of attracting substances since Leber (1) found evidence of a chemotactic agent in rabbit cornea previously irritated with dead staphylococci. He found that leukocytes moved in straight lines from all directions to the irritated area. Since these early experiments, technics have been refined and many substances have been reported to attract leukocytes, including, *inter alia,* bacteria (2), histamine (3), polypeptides (4), starch (5) and sugars (6).

Moreover, various distances of attraction have been reported. For example, staphylococci have been claimed to attract granulocytes from a distance as great as 1.0 mm. (7), while starch attracted granulocytes from a distance of 0.2 mm. (8).

Leukocytes are prone to considerable variations under different physiological conditions. For example, narcotized cells do not exhibit chemotactic behavior. Moreover, some species of virulent bacteria when phagocytosed are capable of destroying the protoplasm of granulocytes (9). Investigators of chemotaxis have emphasized the attracting substance, but have failed to consider the source and physiological status of the granulocytes. The study of chemotactic reactions of granulocytes under carefully controlled conditions should help toward the solution of the mechanisms involved in this phenomenon.

This work was undertaken for the purpose of studying chemotactic behavior of granulocytes obtained from animals maintained under controlled conditions. The neutrophilic granulocytes were the cells considered, but reference is sometimes made to them as leukocytes or white blood cells.

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MATERIAL AND METHODS

A. Material

1. Animals. Adult rats of the Wistar strain weighing between 250 to 350 g. were used throughout the experiments, except in instances where repetition of other investigators' procedures was necessary. The animals were kept in as uniform a condition as possible. In order to avoid undue excitement when being prepared for experiments, the rats were handled frequently. For the same reason, the males were separated from the females.

The diet for the fed animals consisted of Purina dog chow and a semiweekly supplement of fresh green vegetables. Some of the animals were unfed for 36 hours, and others for five to six days before experimentation. When animals were unfed for long periods, they were isolated to prevent cannibalism. Water, however, was available at all times.

2. Plasma. Blood was obtained either from the normal or unfed rat by a heart puncture with a No. 21 gauge sterile needle attached to a 10 cc. Luer syringe which contained 0.2 cc. barium heparinate solution for each 10 cc. of blood (35 mg. crystalline barium heparinate dissolved in 15 cc. normal saline). The needle was removed from the syringe and the blood slowly expelled into a sterile 15 cc. centrifuge tube. The plasma was obtained by centrifuging for five minutes at $6500 \times G$. The supernatant plasma was removed by pipette transfer.

The clotting time of the supernatant heparinized plasma was determined by mixing one drop of the plasma with one drop of thromboplastin solution (see below). The time of formation of the clot was established by picking at the edges of the mixture with a dissecting needle at intervals of 10 seconds until a stringiness was noted. This procedure produced a clot which was not too rigid and therefore offered no obstacle for the migrating granulocytes, and yet had sufficient consistency to prevent dislocation of granulocytes by convection currents. The granulocytes in these preparations exhibited good amoeboid movement.

The plasma used in these experiments had a clotting time of approximately two minutes. This enabled completion of the entire slide-cover-slip preparation before clotting occurred.

3. Granulocytes. In order to obtain the granulocytes, the animals were first injected intraperitoneally with 20 cc. of sterile normal saline. Intraperitoneal injections of saline were performed at the same time of day. No anaesthetics were used, since their narcotizing effects might invalidate the results. Three hours after the intraperitoneal injections of saline, the rat was killed by heart puncture

exsanguination, the peritoneal cavity immediately opened, and the fluid therein removed with a special pipette and transferred to a sterile 15 cc. tube. The special pipette, fitted with a rubber bulb, had a tip opening of approximately 1 mm. This comparatively large opening was used to prevent possible damage to the granulocytes. Exsanguination prevented the contamination by blood of the peritoneal fluid containing the granulocytes.

The granulocytes were studied under two conditions: *a*) free granulocytes; and *b*) agglutinated granulocytes. The free granulocytes were obtained by placing a drop of the suspension from the peritoneal cavity into a drop of plasma within 10 to 15 minutes after collection. For the agglutinated granulocytes, the peritoneal fluid was allowed to agglutinate in the tube by standing at room temperature for approximately one hour. A small portion of the agglutinated mass was separated and placed in the center of the tissue-culture medium.

4. Thromboplastin. Thromboplastin was prepared from the lungs of the same animal from which the granulocytes and plasma were obtained. The lungs, removed under aseptic conditions, were washed several times with sterile saline to remove traces of red cells. The washed lungs were then ground to a pulp in a sterile mortar with an equal amount of sterile, reagent grade sand. Ten cc. of sterile saline were added to the pulp and the mixture ground again. The resulting fine suspension was transferred to a sterile 15 cc. tube and centrifuged for five minutes at 6500 × G. The supernatant solution, which contained the thromboplastin, was used to coagulate the plasma.

B. Technic for Studying Chemotactic Behavior

The method used for studying the activity of granulocytes was a modified combination of the slide-cover-slip procedure used by McCutcheon (7) and the tissue-culture technic worked out by Meier (2) and by Chambers and Grand (6).

The modified procedure was essentially as follows:

1. One drop of plasma was placed on a sterile slide. For nonagglutinated granulocyte preparations, a small drop of granulocyte suspension was added to the drop of plasma and stirred with a sterile needle.

2. In the agglutinated-granulocyte experiments, a small portion of the clumped mass was placed in the center of the plasma drop.

3. The substances to be tested were then added to the granulocyte-plasma medium. Solid particles, such as starch granules of approximately 0.2 to 0.4 mm. in diameter, were sparingly sprinkled over the plasma preparation. The mixture was then gently agitated with a needle in order to coat the particles with plasma. Clotting was then induced with a drop of thromboplastin added to the preparation without agitation, and a sterile cover slip placed over the culture. The cover slip did not press down on the leukocytes since the starch granules acted as a support. After three minutes, the edges of the preparation were sealed with paraffin wax and the slides placed in an incubator maintained at 39° C.

Glass microcapillaries with an outside diameter of approximately 0.1 mm. were employed for the testing of plasma soluble substances on granulocyte movement. The procedure described for the use and preparation of microcapillaries by Chambers and Grand (6) was used.

In all experiments nonattracting, or indifferent, and attracting substances were included. Glycogen invariably attracted leukocytes while physiological saline produced no attraction. Therefore, these two substances were used as controls.

EXPERIMENTAL PROCEDURE AND RESULTS

A. Agglutination of Granulocytes in Peritoneal Fluid

The granulocytes were obtained from animals in three nutritional states: *1*) well fed; *2*) unfed for 36 hours; and *3*) unfed for five to six days. The peritoneal fluid, ranging from 6 to 10 cc., was placed in sterile centrifuge tubes which were plugged with cotton and allowed to remain at room temperature. The changes that occurred in the suspension of granulocytes were the following:

1. Well-fed animals. No definite agglutination of granulocytes was noticed. After several hours most of the cells settled to the bottom of the tube in a loosely adherent state.

2. Animals unfed 36 hours. Definite tendency toward agglutination was noticed. Adhesion of one cell to another often occurred within 15 minutes. The cells massed into a cone shape, suspended in the center of the liquid medium. The entire mass could easily be removed from the tube and when two dissecting needles were used to separate pieces from the agglutinated mass, it was found that the cells adhered firmly to one another and could be separated only with difficulty.

3. Animals unfed five to six days. Tendency toward agglutination was not definite and after two to three hours the granuloyctes clumped in small hemorrhagic clusters which adhered to the sides of the tube and sometimes one large cluster would fall to the bottom.

B. Attraction of Granulocytes by Various Substances

Tissue-culture preparations of granulocytes obtained from animals subjected to various nutritional conditions were made by using solid particles or dissolved substances placed in capillary tubes. Responses of the granulocytes were considered positive when the cells adhered to the solid particles being tested, and after making contact, spread out in a thin layer over the surface of the particle. Small objects engulfed by a single granulocyte

were considered to be phagocytosed. When fluid-filled capillary tubes were used, the entry of granulocytes into open ends of the tubes was considered as a positive response. For substances showing no response, *i.e.*, neither negative nor positive, the granulocytes did not adhere to, nor collect near, nor were they repulsed by, the substances being tested.

Six to eight capillary tubes were used for each preparation. One of the capillary tubes always contained glycogen, a known attracting substance, which acted as an index of the chemotactic behavior of the granulocytes. Whenever the granulocytes moved away from the area of a substance being tested, the behavior was considered a negative attraction. The following results were obtained:

1. Well-fed animals. Many substances failed to attract the granulocytes. Glucose elicited no response, but polysaccharides such as glycogen and starch always attracted the granulocytes. The rate of movement averaged approximately 20 microns per minute. The response of granulocytes to various substances is summarized in Table I.

2. Animals unfed 36 hours. Granulocytes from unfed rats showed more activity and greater rate of movement than those obtained from well-fed rats. The rate of movement averaged approximately 30 to 40 microns per minute. A higher percentage of starved granulocytes moved within a given time and many more pseudopodia were continually extended, giving the cells an irregular shape, instead of the tendency toward rounding, as observed in the apparently well-fed granulocytes.

3. Animals unfed five to six days. No attraction to substances was noted since the granulocytes showed only slight motility. Only a few granulocytes left the clumped mass and moved a distance not exceeding 1 mm. after six hours of incubation.

No differences in behavior were noted between the agglutinated and nonagglutinated granulocytes.

TABLE I

Attraction of granulocytes *

Substances tested	Rats well-fed	Rats unfed 36 hours	Rats unfed 5 to 6 days
1. Starch (potato)	x	x	0
2. Starch (soluble)	x	x	0
3. 3 and 5% Glycogen	x	x	0
4. 3 and 5% Agar agar	x	x	0
5. 3 and 5% Glucose	0	x	0
6. 3 and 5% Sucrose	0	x	0
7. 3 and 5% Lactose	0	x	0
8. 3 and 5% Maltose	0	x	0
9. 3 and 5% Gelatin	0	0	0
10. Protein hydrolysates (acid)	0	0	0
11. Protein hydrolysates (enzyme)	0	0	0
12. Blood plasma	0	0	0
13. 1:1000 Histamine	0	0	0
14. 1:100 Endotoxin from pyrogenic bacteria	x	x	0
15. 3 and 5% Lactic acid	—	—	0
16. 1% Heparin	0	0	0
17. Carbon particles	0	0	0
18. Glass particles	0	0	0
19. Physiological saline	0	0	0

* x indicates positive attraction.
0 indicates no attraction.
— indicates negative attraction.

C. *Movement of Granulocytes toward Starch Grains*

Starch grains were used as the attracting substance. The movement of nonagglutinated granulocytes after one hour of incubation was observed under the microscope equipped with a warm stage. The following results were obtained:

1. Well-fed animals

a) Granulocytes showed no directional movement except when reaching a distance approximately 0.1 mm. from the periphery of the granule. Even within this area, many of the granulocytes reached the surface of the starch granule by random movement, while others showed a definite directional motion toward the attracting substance. When nonattracting substances such as carbon particles were used, directional motion was not observed at any distance from the surface of the carbon. When soluble starch was used, the area of attraction was found to be 0.7 to 1.0 mm. from the surface of the starch granule. Rate of movement was from 10 to 25 microns per minute.

b) Granulocytes on reaching the surface of the starch granule either remained there and spread out, or else moved away from the granule.

c) Starch granules too large for phagocytosis were completely coated by a monolayer of granulocytes. Other granulocytes, on reaching the coated starch grains, moved away. Granulocytes which formed the monolayer would often detach themselves from the surface of the attracting substance. This happened after several hours of incubation. Other granulocytes would then occupy the un-

covered areas of the starch. There was little adhesion of one granulocyte to another.

d) When the starch granules were removed from the preparation after the completion of the experiment and studied under high power, it was noted that small grooves were present on the originally smooth surfaces. Grooves were not apparent in similar preparations containing no granulocytes.

2. Animals unfed 36 hours. A monolayer of granulocytes was also produced on the starch grains, but when other granulocytes came into contact with the cells forming the monolayer, adhesion occurred, thereby resulting in the accumulation of a large mass of cells. Other observations were similar to those obtained in the experiments on the attraction of granulocytes by various substances.

3. Animals unfed five to six days. Results were similar to those on attraction of granulocytes by various substances.

D. Movement of Granulocytes from a Clumped Mass

Six capillary tubes filled with soluble material were used for each preparation. The positions of attracting and nonattracting substances were varied. For example, in some preparations three nonattracting capillary tubes were placed on one side of the granulocyte mass, and three attracting substances on the other. In other preparations, both attracting and nonattracting capillary tubes were placed on one side. In some, the attracting capillary tube was flanked by two nonattracting capillaries; in others, one nonattracting capillary was flanked by two attracting capillary tubes. After one hour of incubation, the movements of agglutinated granulocytes were observed on a warm stage. The following results were obtained:

1. Well-fed animals

a) The rate of movement was the same for both attracting and nonattracting substances.

b) The granulocytes always moved away from the clumped mass so that a directional movement existed whether an attracting substance was present or not.

c) After six to eight hours a complete circle of outwandering cells was found, extending 4 to 6 mm. away from the periphery of the clumped mass.

d) The attracting substances showed no greater influence on the directional movement than did the nonattracting substances.

e) Granulocytes, on reaching the surface of the particular attracting substance, either remained there, spread out in a thin layer, or moved away. On the other hand, the granulocytes never spread out on the nonattracting particles, but always moved away. In nearly all instances granulocytes collected in large numbers inside the capillary tubes which contained attracting substances.

2. Animals unfed 36 hours. The results were essentially the same as with granulocytes obtained from well-fed animals except that the movements were more rapid (35 to 40 microns per minute), more pseudopodia were present and the granulocytes became massed around the attracting substances.

3. Animals unfed five to six days. The granulocytes showed no tendency toward motion, and after six hours only a small number of cells had left the clumped mass, and these had traveled only a distance of approximately 1 mm. Furthermore, the number of granulocytes was smaller than from well-fed and 36 hour unfed rats, and red cells were always found intermingled with the white cells.

DISCUSSION

The experiments described in this paper offer a clue to the results of Chambers and Grand (10) and the discrepancy presented by McCutcheon (7) in his review. Sheldon, in a personal communication, cited by McCutcheon, did not state the condition of the animals used as the source of his material. The results reported in this paper would indicate that the animals, as used by Sheldon, were not starved. Chambers and Grand, however, used as their culture medium the plasma from roosters which had been starved for 48 hours for the purpose of eliminating fat from the plasma.

The question now encountered is: Why do polysaccharides attract granulocytes from both well-fed and starved animals while only the latter are attracted by mono- and disaccharides? Granulocytes from the starved animals are attracted by glucose, other sugars and polysaccharides *in vitro*, but granulocytes from well-fed animals show attraction for polysaccharides only.

Granulocytes seem to be attracted preferentially by polysaccharides, since granulocytes from both starved and well-fed animals are attracted to glycogen and starch. This may suggest a metabolic

preference. Glucose is always present, although in variable amounts, in the blood stream. It is only when the store of glucose in the blood stream has been lowered to the starvation level (approximately 70–80 mg.%) that the granulocytes are attracted by glucose *in vitro*. The problem concerning the preference of granulocytes for polysaccharides may also be attacked from the point of view of energy and metabolism. Polysaccharides probably present a more economical pathway for the cells. Polysaccharides such as glycogen and starch are more directly fermentable since phosphorolysis does not depend upon a high accumulation of adenosine triphosphate (11).

That nutritional environment plays a role in immunity has been reported by Sprunt (12) who has shown that prolonged fasting may cause a tenfold increase in the normal antiviral resistance in rabbits. Richardson (13) determined the phagocytic power of leukocytes of diabetic patients and depancreatized cats with acidosis or those with uncontrolled diabetes. In both situations, a significant decrease occurred in the phagocytic power. Also, one of the first reactions of the body toward infection and fever is a loss of appetite which might starve granulocytes, thus making them more active. In diabetics with high blood sugar, granulocytes have an abundant supply of glucose. The experiments in this paper indicate that granulocytes from well-fed animals are slow-moving and less "sticky" than the granulocytes from unfed animals. Furthermore, the results show that granulocytes from starved animals move more rapidly and are much more adhesive to one another. Granulocytes from well-fed animals frequently move away after making contact with either glycogen or starch, which seem to be the preferred nutritional substances. A granulocyte from a fasting animal, however, adheres to glycogen or starch, spreads over the surface of these substances and, if the particles are small enough, completely ingests them. Kuna, Blattberg and Reiman (14) showed that leukocytes obtained from rats well-fed for four weeks prior to a 36 hour period of starvation, when compared with leukocytes from continuously well fed animals, showed twice the percentage of phagocytosing white blood cells which engulfed three times the number of bacteria.

Some investigators have shown an increase and others a decrease in the phagocytic index with a reduction of food intake. Mills and Cottingham (15) found a relationship between protein consumption and phagocytosis. Rats lost weight the first week on a deficient diet, but the blood phagocytes did not become less active until the second week, and the lowest level of phagocytosis was reached at the end of the fourth week.

In rats fed a grossly deficient diet, Berry, Davis and Spies (16) measured a decrease in the phagocytic activity of neutrophiles as compared to that in animals on an adequate diet. Columbo (17) noted a moderate increase in complement accompanying malnutrition in children. The phagocytic titers were frequently elevated. Berry, Davis and Spies (18) noticed that in cases of all types of anemia an increase in neutrophilic activity was found and the magnitude of the effect was roughly proportional to the severity of the anemia. Berry, Leyendecker and Spies (19) also found increased phagocytic activity of neutrophiles in blood, measured *in vitro*, of patients with anemia, many of whom were malnourished. As stated by these authors, "Such an apparent increase in phagocytic activity in anemia was particularly surprising, since many of the persons whose blood was studied were malnourished." More recently, Gordon and Katsh (20) have shown that chronic starvation, induced in normal animals, resulted in a greatly increased uptake of thorium dioxide by the spleen.

Some of the contradictions in the literature may be explained by defining the conditions under which the experiments were performed. Activity of neutrophiles and phagocytosis decreases with undernourishment, *i.e.*, elimination of one or more essential nutritional elements for an extended period of time. Phagocytosis and neutrophile activity are increased in an animal which had been maintained in a good nutritional state prior to a 36 hour starvation period. The term "subnutritional phagocytosis" has been suggested for this phenomenon (21). However, if the starvation period of a previously well-nourished animal is extended to five or six days, the neutrophile activity decreases to almost nil with consequent decrease in phagocytosis possibly resulting in greater susceptibility to infection.

Further evidence can be presented to show that the action of granulocytes is subject to environmental conditions. It is possible that one of the

main factors concerning this attraction of granulocytes to bacteria is that bacteria are frequently coated with polysaccharides. A negative chemotaxis may result if bacteria coated with polysaccharides also produce toxins which are harmful to the granulocytes. However, when such toxins are neutralized by antibodies, the granulocytes are attracted to the bacteria (22).

Preliminary work in this investigation on the attraction of granulocytes by acids has shown that a pH below 6.7 causes a negative attraction or death if the granulocyte cannot move away. Thus, it seems that granulocytes avoid a harmful environment and accumulate in areas containing preferred metabolic substances, such as glycogen. Lactic acid, which is detrimental to leukocytic activity, repels and, conversely, glycogen, which is a probable nutrient for granulocytes, exhibits positive attraction.

The mechanisms involved in granulocytic migration are varied. If the starch granule (too large to be phagocytosed) was used as the attracting substance, it was found that attraction for granulocytes occurred 0.1 to 0.2 mm. from the surface of insoluble starch. However, soluble starch showed an area of attraction ranging from 0.5 to 0.7 mm. This suggests that a concentration gradient may be a factor in attracting granulocytes toward starch. It is only within the zone of a concentration gradient that granulocytes move toward the starch grain. Elsewhere the movement is random. Granulocytes from well-fed animals did not always show the attraction for starch grains even within the concentration gradient zone. Some moved at right angles to the substance and some even moved away from the substance.

Granulocytes from unfed animals showed a decided movement toward the starch granule when within the concentration gradient zone. After a few hours when the granulocytes moved away, some erosion of the starch grains could be seen, suggesting a possible utilization of some starch by the granulocytes. This phenomenon was observed by both authors independently.

Experiments in this paper have shown that the movements of granulocytes from an agglutinated mass is always in a definite direction away from the mass. This directional movement is the same whether an attracting or a nonattracting substance is present. Moreover, if a capillary tube is placed tangentially to the agglutinated mass, the granulocytes will collect on the sides of the capillary because their motion has been impeded by this obstruction.

It seems that the true indication of attraction may not only be the contact of the substance by the granulocytes, but also the adherence and the spreading of the granulocytes over the substance.

It is evident that agglutination of granulocytes varies with the nutritional state of the animals from which the granulocytes were obtained. Thus granulocytes from well-fed animals started to adhere to the walls of the tube, but, since they were not sticky, fell to the bottom. From animals unfed for 36 hours, the granulocytes were "sticky" and formed complete cone-shaped masses in the center of the liquid. No adhesion to the sides of the tube was noted. From animals unfed for five to six days, the agglutination was less evident, but individual masses of granulocytes intermixed with erythrocytes adhered to the sides of the tube. The clumped mass from the 36 hour-unfed animals was the hardest to separate into smaller pieces. It seems probable that moderate starvation rendered the granulocytes sticky so that they adhered very easily to nutritive substance. This may account for the accumulation around an attracting substance.

Glycogen seems to be the most likely substance for the attraction of granulocytes. Irritations which produce a tissue breakdown liberate, among other things, glycogen. The insertion of a piece of sterile steel subcutaneously could liberate glycogen from broken cells and thus promote attraction for granulocytes almost immediately. Since only very small quantities of glycogen are required for attraction, it is possible that exudates from inflammations do contain glycogen and that glycogen may be the active principle and not necessarily a polypeptide, as postulated by Menkin (23). Many simple polypeptides which have been tested did not attract granulocytes.

The phenomenon of sub-nutritional resistance apparently occurs in previously well-fed animals deprived of food for 36 to 48 hours, with a blood sugar level between 70 and 80%. In diabetics it does not suffice to reduce blood sugar to so-called normal levels of 120 to 140 mg. % to increase resistance. It is necessary to lower the blood sugar to the fasting level of 70 to 80 mg. % in order to produce increased leukocyte activity. Thus a dia-

betic treated pre-operatively with sufficient insulin to lower the blood sugar to a fasting level should show greater resistance to infection.

The experimental evidence presented indicates that it is the granulocytes as well as the substance which must be considered in all types of granulocyte activity. Reaction to the environment by the cells may very well be the key to the mechanisms involved in their action.

SUMMARY

1. Methods utilizing tissue-culture procedures were developed for studying the behavior of granulocytes obtained from rats subjected to varying degrees of underfeeding.

2. Granulocytes obtained from rats unfed for 36 hours had a stronger tendency to agglutinate than those obtained from rats unfed for five to six days. However, granulocytes from well-fed rats did not agglutinate to any appreciable extent.

3. Starch particles too large to be ingested were frequently coated by a single layer of granulocytes obtained from well-fed rats. Granulocytes obtained from rats unfed 36 hours showed a tendency to form many layers around the starch grains.

4. Starting with a clumped mass of granulocytes, the individual cells obtained from well-fed rats always moved away from the clumped mass, regardless of the presence of attracting or nonattracting substances nearby. Granulocytes obtained from rats unfed for 36 hours behaved similarly, except that their movements were more rapid. By contrast, granulocytes from rats unfed for five to six days rarely moved to any appreciable extent from a clumped mass.

5. Granulocytes from well-fed rats were always attracted by glycogen or starch, but not by mono- or disaccharides. Granulocytes from animals unfed for 36 hours were attracted by polysaccharides as well as by mono- and disaccharides. No appreciable attraction of granulocytes was elicited by starch when these cells were obtained from rats unfed five or more days.

6. The possibility that glycogen is the natural granulocyte-attracting agent released on injury of tissue is discussed.

7. Movement of granulocytes was considered random at all times except in instances where granulocytes from rats starved for 36 hours were within a possible concentration gradient zone of an attracting substance.

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ALTERATIONS IN THE IONIC COMPOSITION OF ISOTONIC SALINE SOLUTION INSTILLED INTO THE COLON [1]

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Relatively little is known about the transport of electrolytes across the colonic mucosa, but it has generally been assumed that the colon plays no specific part in maintaining the body's electrolyte equilibrium (1). In 1921 Goldschmidt and Dayton (2, 3, 4) reported that the passage of chloride in and out of the colon depends solely on the concentration of chloride in the luminal fluid. Some years later DeBeer, Johnston and Wilson (5) noted a high concentration of bicarbonate and a raised pH in a small amount of fluid obtained from an isolated colonic loop. Recent studies of the human colon have demonstrated a preferential absorption of chloride over sodium (6, 7).

To evaluate further the role of the colon in electrolyte and acid-base equilibrium a study has been made of the changes in the composition of isotonic sodium chloride solution instilled into the isolated colon of the dog. The data obtained indicate that concentration gradients for chloride, bicarbonate, sodium and potassium are maintained across the colonic mucosa.

METHODS

Fourteen experiments were done in six adult dogs whose colons had previously been isolated by a modification of the technique of Larson-Bargen (8). In this procedure the ileum is transected as close to the cecum as possible, the recto-sigmoid colon is transected as far down in the pelvis as possible, and a side-ileo-end colostomy is done. After the distal ileal stump has been turned in, the proximal colonic segment is exteriorized through a left flank stab wound. At no time was more than 3 cm. of ileum left. These manipulations allowed a blind pouch to be made consisting of the proximal three-fourths of the colon, keeping most of the blood-supply and nerve-supply intact. To avoid overdistention during subsequent experiments, the capacity of each loop was measured under direct vision at operation and was found to be 150–200 cc. The function of the loop was studied beginning three weeks after the operation and over a period not exceeding 16 weeks. During this time the loops were irrigated three or four times a week with isotonic sodium chloride solution. Previous estimates of the viability of this type of preparation and its usefulness in studying colonic function have been made by Johnston (9), who found no perceptible change in the physiology or histology of the colon after as long as seven months. Peristalsis and absorption of glucose and sodium chloride solutions of various concentrations were unchanged during this period (9). In the present studies, punch biopsies from various parts of the pouch taken at intervals showed no histologic abnormalities; moreover, no diminution in capacity occurred. Autopsy of the four animals used in these experiments confirmed the complete isolation of the colonic pouches and did not reveal fistulae.

Experiments were performed by instilling 150 to 200 cc. of isotonic 155 mM. sodium chloride solution through an indwelling, double-ballooned, multi-eyed, Foley-type catheter. Complete closure of the colonic stoma was achieved by placing one balloon just inside the pouch and the other just outside. Specimens were collected in the initial shorter experiments at 15 to 30 minute intervals, but in the later experiments collections were made every one or two hours. The experiments were ended when no further fluid could be recovered. Fluid was withdrawn by unclamping the indwelling catheter and allowing a free flow. The final specimen was recovered by deflating both balloons and inserting the catheter to the full length of the pouch, to drain any fluid pocketed off by segmentation. All specimens were collected and centrifuged under oil. The clear supernatant was analyzed for carbon dioxide content, sodium, potassium and chloride and pH by methods described previously from this laboratory (10). In most experiments the pH of the colonic fluid and the serum concentrations of sodium, potassium, chloride and carbon dioxide were determined several times.

In addition to the above experiments, four acute studies were done on healthy dogs under Nembutal anesthesia by Goldschmidt's (2) method of isolating the colon.

RESULTS

Tables I and II show the changes in colonic fluid and plasma in two representative examples of

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[2] Markle Scholar in the Medical Sciences.

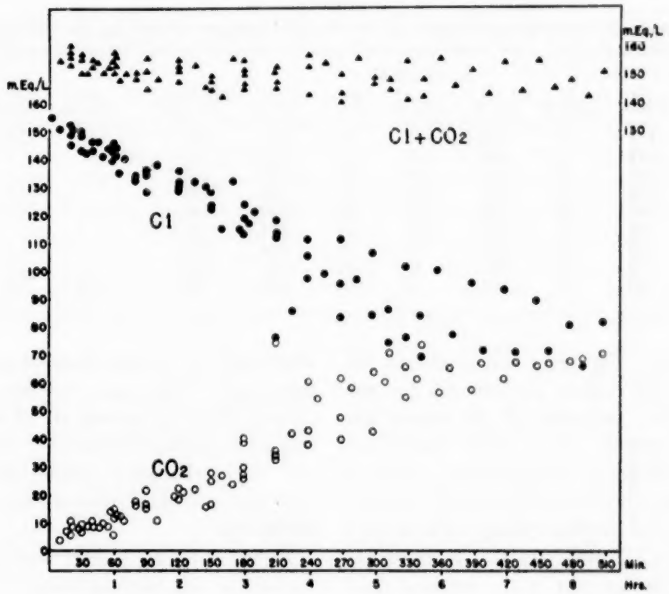

FIG. 1. CHANGES IN ANION COMPOSITION OF ISOTONIC SALINE SOLUTION INSTILLED INTO THE ISOLATED COLON OF THE DOG (14 EXPERIMENTS)

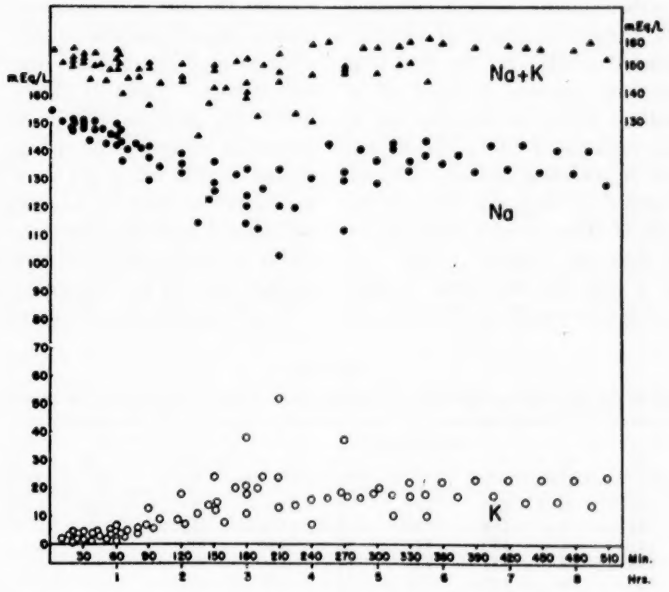

FIG. 2. CHANGES IN CATION COMPOSITION OF ISOTONIC SALINE SOLUTION INSTILLED INTO THE ISOLATED COLON OF THE DOG (14 EXPERIMENTS)

TABLE I

Changes in the electrolyte composition of isotonic saline solution instilled into the colon of a dog

Spec. No.	Time, mins.	Colonic fluid Na mEq./L	K mEq./L	Na+K	Cl mEq./L	CO_2 mEq./L	Cl+CO_2	Na+K+ Cl+CO_2	Serum Na mEq./L	K mEq./L	Cl mEq./L	CO_2 mEq./L
1	0	155	0	155	155	0	155	310	146	5.0	114	19.5
2	60	148	1	149	147	6	153	302				
3	120	140	7	147	130	19	149	296				
4	180	134	11	145	119	27	146	291				
5	210	135	13	148	114	33	147	295				
6	240	131	16	147	106	38	144	291				
7	270	130	17	147	96	48	144	291				
8	300	129	18	147	85	64	149	296				
9	330	133	17	150	77	66	143	293	152	5.0	116	17.5

the 14 experiments. Figures 1 and 2 present the data for anions and cations respectively for the entire group. For purposes of discussion the carbon-dioxide content has been considered equivalent to bicarbonate concentration, since at the final pH range observed (7.6 to 8.0) the calculated carbonic acid fraction (using a pK of 6.1) was no more than 1–3 mM. per L. In most experiments 40 to 60 per cent of the instilled fluid was recovered. Neither the rate of absorption nor the total amount of fluid absorbed affected the composition of the fluid recovered. In all experiments chloride concentration in the luminal fluid declined and the carbon dioxide content increased reciprocally. The carbon dioxide and chloride concentrations changed steadily for the first four to five hours and then appeared to level off at chloride concentrations of 70 to 90 mEq. per L and carbon dioxide contents of 50 to 70 mM. per L. The reciprocal relationship between chloride and bicarbonate tended to keep the sums of the final concentrations of these anions equal to, or only slightly less than, the original values. As shown in Tables I and II, the initial serum-chloride concentration was usually moderately elevated and the carbon dioxide content moderately depressed. This was probably due to the mild chronic diarrhea present in all animals as the result of the ileosigmoidostomy. There were usually only slight changes in serum carbon dioxide content and chloride concentration during the experiments.

Figure 2 shows sodium and potassium concentrations in the luminal fluid. Sodium concentration fell to final levels ranging between approximately 110 and 140 mEq. per L. The changes in sodium were accompanied by a rise in potassium concentration, usually to 15 to 20 mEq. per L. As a result, the sum of the concentrations of measured cations stayed constant or fell moderately. After three to four hours there was no further significant change. Table II shows an experiment in which the greatest fall in sodium and rise in potassium occurred, the sodium concentration declining to 103 mEq. per L while the potassium concentration rose to 52 mEq. per L. Serum sodium and potassium concentrations in all experiments in which they were measured were normal initially and did not change appreciably.

Total osmoles, as calculated from the sum of

TABLE II

Changes in the electrolyte composition of isotonic saline solution instilled into the colon of a dog

Spec. No.	Time, mins.	Colonic fluid Na mEq./L	K mEq./L	Na+K	Cl mEq./L	CO_2 mEq./L	Cl+CO_2	Na+K+ Cl+CO_2	Serum Na mEq./L	K mEq./L	Cl mEq./L	CO_2 mEq./L
1	0	155	0	155	155	0	155	310	145	5.2	108	19.5
2	30	151	3	154	150	8	158	312				
3	60	143	7	150	142	13	155	305				
4	90	138	13	151	136	22	158	309				
5	120	133	18	151	132	22	154	305				
6	150	126	24	150	123	28	151	301				
7	180	115	38	153	116	40	156	309				
8	210	103	52	155	77	75	152	307	141	5.4	113	20.5

sodium, potassium, chloride and carbon dioxide content, occasionally remained unchanged but usually fell slightly.

In the four acute experiments done under Nembutal anesthesia, the changes in carbon dioxide content, chloride, sodium and potassium were similar to those observed in the chronic experiments. In four additional experiments carried out following marked reductions in the bacterial flora as the result of antibiotics given orally and by infusions in the loop, the changes were also comparable to those shown in Figures 1 and 2.

DISCUSSION

The data demonstrate that concentration gradients for chloride and bicarbonate are maintained across the colonic mucosa. These cannot be accounted for by a simple Donnan equilibrium expression but could be explained by postulating that the colonic mucosa secretes bicarbonate into the lumen or actively absorbs chloride into the plasma in an exchange for bicarbonate. If, on the other hand, there were any source of bicarbonate ion inside the colon, the findings might also be explained without invoking an active transport of either bicarbonate or chloride. Splitting of urea by bacteria in the large bowel produces bicarbonate and ammonium and may serve as a continuing source of bicarbonate ions. The constant generation inside the colon of bicarbonate and its subsequent diffusion into the plasma could establish and maintain concentration differences of the observed magnitude. Although from the present data it is not possible to choose between the alternatives of active transport and rapid generation of bicarbonate as the mechanism primarily responsible for the observed changes in anion composition, there are several facts which suggest that ion secretion may be the fundamental factor. Firstly, although the experiments were carried out in many animals the rates of change in the bicarbonate and chloride concentrations and the final levels were remarkably constant. In addition, marked reduction in, and alteration of, the bacterial flora after antibiotic administration did not alter the pattern of response significantly. The fact that there was usually little or no depression of the sum of the concentrations of sodium and potassium implies that there was no large accumulation of the ammonium ion. This hypothesis has been confirmed by analyses for ammonium in several experiments (11) and indicates either that ammonium was reabsorbed at a far more rapid rate than bicarbonate produced simultaneously or that urea splitting could not account for the elevated bicarbonate concentrations. Definitive evidence for active transport would require sterilization of the colonic loop, a condition which has not been achieved in these studies.

If the colons in dog and man behave similarly, the present observations would seem to account for, at least partly, the development of hyperchloremic acidosis after ureteroenterostomy (12–14). Regardless of whether the changes in the composition of the fluid in the lumen are due to a loss of bicarbonate from the plasma in exchange for chloride or to the absorption of chloride and ammonium from the lumen, the effect on the acid-base equilibrium would be the same. The clinical finding that urinary stasis in the colon increases the severity of the acidosis in patients with ureteroenterostomy (12, 13) is consistent with the present observations of progressive change in the instilled solution during the first four to five hours of study. The degree of acid-base disturbance which ultimately results probably depends in part on the integrity of renal function.

The data also indicate that in many instances concentration gradients for sodium and potassium, not explainable by a simple Donnan equilibrium expression, are maintained across the colonic mucosa. This is probably due either to secretion of potassium or to active absorption of sodium. The accumulation of ammonium might explain in part the depression of sodium concentration when the latter was unaccompanied by a reciprocal rise in potassium but would not explain the increased potassium concentration. The relationship of the elevated potassium levels in the colonic fluid to the high potassium concentrations in diarrheal stools requires further study.

A consideration of total osmoles as calculated from sodium, potassium, chloride and carbon dioxide content would indicate that the osmotic pressure of the original solution stays approximately the same or drops slightly. It has been shown by Diena (15) and Goldschmidt and Dayton (4) by measurement of freezing point depression that colonic contents are in osmotic equilibrium with blood. It would appear likely, therefore, that the

apparent reductions in osmolarity calculated from some of the present data are due to the accumulation in the colonic fluid of unmeasured solutes.

SUMMARY

Following instillation of isotonic sodium chloride solution into the isolated colon of the dog, gradients are established across the colonic mucosa characterized by a higher concentration of bicarbonate and lower concentration of chloride in the colon than in the plasma. The sum of the concentrations of these anions in the colonic fluid remains essentially unchanged. The development of this equilibrium probably plays a part in the genesis of hyperchloremic acidosis after ureteroenterostomy. In most instances, concentration gradients across the colonic wall are also established for sodium and potassium.

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THE EFFECT OF CORTISONE ON THE URINARY EXCRETION OF 17-KETOSTEROIDS IN PATIENTS WITH CUSHING'S SYNDROME [1]

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INTRODUCTION

The administration of cortisone to patients with virilism associated with adrenal hyperplasia results in a marked decrease in urinary 17-ketosteroid excretion (1–3) whereas no significant fall occurs in those patients whose virilism is due to an adrenal tumor (4, 5). The administration of cortisone inhibits pituitary adrenocorticotropin secretion causing secondary atrophy of the adrenals (6, 7); hence a decrease in 17-ketosteroid excretion ensues in those patients in whom the normal pituitary-adrenal interrelationship is preserved.

The data obtained in patients with adrenogenital syndrome (4) as a result of the injection of cortisone stimulated a similar investigation of patients suffering from an allied disease, Cushing's syndrome. It is generally agreed that the manifestations of Cushing's syndrome are due to excessive 11-oxygenated corticosteroid secretion by the adrenal cortex, although the pituitary (8) and the hypothalamus (9) as well as the adrenal (10) have been postulated as the site primarily involved.

If either an adrenal or pituitary tumor is present, it seems reasonable that the source of excessive secretion responsible for the disease picture lies in the neoplastic tissue and in such patients because of the autonomous nature of the tumor no fall in 17-ketosteroids would be anticipated following the injection of exogenous cortisone. On the other hand if no tumor exists, the bilateral adrenal hyperplasia may arise *de novo* and be independent of pituitary control, or the normal pituitary-adrenal interrelationships may still be operating. If the latter obtains, then the administration of cortisone should result in a fall in the 17-ketosteroid excretion.

Since the levels of urinary 17-ketosteroid excretion in Cushing's syndrome are considerably lower than those encountered in the adrenogenital syndrome the results following the administration of cortisone would of necessity be less striking. However, if significant decreases in 17-ketosteroid excretion could be demonstrated, the administration of cortisone might provide a physiological method for determining whether the hyperplastic glands found in Cushing's syndrome are under pituitary influence. The present report describes the responses in urinary 17-ketosteroid excretion of 6 patients with Cushing's syndrome during four to five days' administration of large doses of cortisone.[3]

METHODS AND MATERIALS

A brief summary of the case histories of the 6 patients follows:

Patient 1. C. C. was a 29 year old woman with hypertension (160/100), amenorrhea, decreased glucose tolerance, demineralization of the spine, buffalo obesity, striae, acne and paranoid reactions. In April 1951, the pituitary was irradiated with a total tumor dose of 3350 R with only temporary improvement. In March of 1952, the present studies were conducted and subsequently a subtotal bilateral adrenalectomy was performed and the diagnosis of bilateral adrenal hyperplasia confirmed. Postoperatively the patient improved.

Patient 2. S. K. was a 20 year old male exhibiting all the manifestations of Cushing's syndrome—characteristic obesity, blood pressure of 206/140, decreased glucose tolerance, multiple purple striae. A right partial adrenalectomy was performed in July 1951, with removal of 6.5 gms. of hyperplastic gland. Subsequently, pituitary irradiation in September 1951, totalling 4500 R tumor dose, resulted in a temporary partial remission. The first cortisone study (2a) here reported was undertaken in April 1952.

[1] Aided by grants from the American Cancer Society as recommended by the Committee on Growth, National Research Council and the United States Public Health Service.

[2] Fellow, American Cancer Society.

[3] The cortisone used in this study was kindly supplied by Dr. Elmer Alpert of Merck, Inc.

Since the results of the cortisone test were not decisive it was repeated in October 1952 (2b). On November 7 1952 the right adrenal gland was removed *in toto*.

Patient 3. J. J. was a 29 year old policeman with documented Cushing's syndrome—facial obesity, blood pressure of 190/139, frank diabetes, severe acne, purple striae, compression fracture of the ninth thoracic vertebra and confused mental state. The cortisone study was performed April 1952. Subsequently, in the course of three operations, the greater portion of adrenals has been removed and no evidence of a tumor has been demonstrated. Postoperatively no adrenal substitution therapy was needed. One month after the third operation, pituitary irradiation (totalling 4000 R tumor dose) was given and at present, two months after completion of X-ray therapy, the patient is not improved.

Patient 4. A. G. was a 30 year old housewife with Cushing's syndrome exhibiting rounding of the face, mild hypertension (140/90), amenorrhea, diabetic glucose tolerance curve. Subsequent to the present study with cortisone, a unilateral adrenalectomy in August 1952 established the diagnosis of adrenal hyperplasia.

Patient 5. H. H. was a 38 year old housewife with Cushing's syndrome manifested by moon-facies, buffalo hump, easy bruisability, mild hypertension (150/90) and a diabetic glucose tolerance curve. She had no roentgenological evidence of osteoporosis and her menses were normal. A left unilateral adrenolectomy was performed on January 14, 1953 and a hyperplastic adrenal weighing 12 gms. was removed.

Patient 6. C. R. was a 26 year old salesman whose Cushing's syndrome ran a fulminating course. His findings included rounding of the face, blood pressure of 190/120, diabetes, purple striae, acne, confused mental state with toxic psychosis. A left adrenal carcinoma was removed at operation in December 1951. Two months later, metastases were discovered in the lungs and spine. The cortisone study here reported was undertaken in February 1952. Due to the rapid mental and physical deterioration of the patient, an hypophysectomy was performed by Dr. Lawrence Pool of the Neurological Institute. The patient subsequently died of carcinoma and sections of the contents of the sella turcica revealed no pituitary tissue.

RESULTS

Adrenal hyperplasia. The 5 patients with proven adrenal hyperplasia were each injected with 200 mg. of cortisone intramuscularly daily for four or more days (Table I). In all 5 patients, there was a significant decline in the excretion of urinary 17-ketosteroids. In 1 patient, S. K., the decline in 17-ketosteroids as a result of cortisone therapy was only 25 per cent (2a). However, when the experiment was repeated six months later an impressive reduction in 17-ketosteroid excretion (2b) was noted. There were no deleterious effects of the administered cortisone on any of the patients.

TABLE I

The effect of cortisone on urinary 17-ketosteroid excretion in patients with Cushing's syndrome

Patient No.	1	2		3	4	5	6	7
		a	b					
Day before cortisone								
4			16.6					
3	14.3	22.1	13.8		23.8		51.6	
2			14.2	29.9		19.2	40.8	21.2
1	9.0	16.6	12.9	25.9	22.9	20.2	39.3	17.0
on cortisone *100–200 mg/day*								
1	6.3	13.8	12.9		11.2		53.1	12.2
2	5.8	14.2	14.0		8.0	11.4	56.1	
3		12.6	5.8	22.9	11.0	14.8	63.0	13.4
4	3.6	13.1	6.3	23.6	13.5	12.4	59.3	
5				19.2		11.3		

Patients 1 to 5: adrenal hyperplasia
Patient 6: adrenal carcinoma
Subject 7: normal male

Adrenal carcinoma. It has been reported that Cushing's syndrome due to adrenal carcinoma may be accompanied by a wide variation in the levels of 17-ketosteroid excretion, but that the amounts of these substances are usually elevated (11). In the patient here reported the control values were definitely, though not extraordinarily, elevated. The administration of 100 mg. a day of cortisone for four days intramuscularly was without demonstrable effect upon the 17-ketosteroid output (Table I, No. 5). Apparently the tumor in this patient was autonomous and, unlike normal or hyperplastic adrenal tissue, was not under pituitary control. Hence pituitary inhibition as achieved by cortisone administration was without effect upon the secretion of 17-ketosteroid precursors. This was further borne out by the fact that following the complete hypophysectomy the level of urinary 17-ketosteroids and the clinical course remained unchanged.

Normal individuals. It has been amply demonstrated that when therapeutic doses of cortisone are administered to normal individuals there is a fall in 17-ketosteroid excretion. Thus, as can be seen from the table (No. 7), the administration of 200 mg. per day of cortisone in a normal male resulted in a 40 per cent decline in total neutral 17-ketosteroids.

DISCUSSION

It is not clear whether the physiological manifestations of Cushing's syndrome result from excessive amounts of adrenocorticotropin stimulating the adrenal glands or whether the overproduction of corticosteroids arises spontaneously within the adrenals. There are several indications from available clinical material which suggest that the first of these possibilities is the more commonly encountered abnormality: *1*) Adenomas of the pituitary occur in 50 per cent of the cases while adrenal tumors, including benign and malignant types, are found in less than 30 per cent of such patients (12), *2*) irradiation of the pituitary results in some degree of improvement in at least 30 per cent, and *3*) both adrenal cortices show hyperplasia in a high per cent of the cases (58 per cent) (12). As mentioned above, there is ample evidence that one of the physiological actions of cortisone or of compound F is to suppress ACTH secretion by the adenohypophysis (6, 7, 13). This has been demonstrated in the virilizing syndrome (1–4). The fall in urinary 17-ketosteroids which occurs in Cushing's syndrome as a result of cortisone administration is in all probability similarly mediated. The present findings provide evidence for the speculation that the hyperplastic adrenal glands in Cushing's syndrome remain under pituitary control since, when ACTH secretion is suppressed by cortisone, the concomitant fall in urinary 17-ketosteroid secretion reflects a decreased cortical activity. Such a fall did not occur in the one patient with an adrenal carcinoma. The presumed suppression of his pituitary ACTH secretion by cortisone was without effect since the great bulk of the precursors of the 17-ketosteroids was provided by the autonomous tumor which was free of pituitary control. This was subsequently confirmed when complete hypophysectomy failed to alter the 17-ketosteroid values.

These results bring up another point of speculation. If suppression of adrenocortical activity occurs as a result of exogenous cortisone or compound F administration (4) and there is a resultant decline in urinary 17-ketosteroids, the adrenal must be secreting other steroids which are 17-ketosteroid precursors besides cortisone and compound F. The latter are known to be degraded and excreted as 17-ketosteroids to a certain extent (14, 15). It is possible that the adrenal contribution to the 17-ketosteroids includes an androgen. Assay of cow adrenal vein blood has demonstrated the existence of an adrenal androgen (16).

SUMMARY

The urinary excretion of 17-ketosteroids in 6 patients with Cushing's syndrome has been studied during a four to five day period of exogenous cortisone administration.

No alteration was observed in the abnormally high excretion of 17-ketosteroids in 1 patient with a metastatic adrenal tumor.

Significant decreases in 17-ketosteroid levels were noted in 5 patients with proven hyperplastic adrenal cortices.

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THE EXCHANGEABLE THYROID HORMONAL POOL. I. ITS MAGNITUDE AND RATE OF TURNOVER IN VARIOUS THYROID STATES IN MAN [1, 2]

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Recent reviews (1, 2) have summarized the significant advances in the elucidation of certain aspects of thyroid physiology resulting from studies utilizing radioactive iodine and improved chemical methods for the determination of iodine. Quantitative data defining the size of the thyroid hormonal pool and the rate and amount of "peripheral utilization" or turnover of the thyroid hormone within this pool are, however, fragmentary (1).

Estimates based on the thyroxine requirements in man at various basal metabolic levels indicate that approximately 10 mgm. of thyroxine are stored in the human body (3). Values obtained by various methods for the daily thyroid hormone turnover in man are listed in Table I. Most recently, Riggs (1) has estimated that the half-life of thyroid hormone in tissues is 11.9 days.

This report is concerned with the experimental determination of the magnitude and turnover of the exchangeable thyroid hormonal pool in man based on the rates of disappearance from the circulation of endogenously labelled (I^{131}) human thyroid hormone after its infusion into patients with normal and abnormal thyroid function.

MATERIALS AND METHODS

Seven patients with the classical symptoms, signs, and laboratory findings of thyrotoxicosis served as donors of endogenously labelled thyroid hormone. Each donor received 10.3 to 23.1 millicuries of I^{131} for therapeutic purposes. The dosage was calculated according to a previously described method (12) and delivered 5 to 15,000 R.E.P. to the donors' thyroid glands. Two to six days later blood was withdrawn from each donor under sterile precautions into acid citrate solution. After centrifugation for 30 minutes at 3,000 R.P.M., the plasma was separated under sterile conditions. Analysis by trichloracetic acid precipitation (13) and after dialysis showed 100 per cent protein-binding in five of the seven donors; the plasma iodine of the remaining two donors was 80 and 84 per cent protein-bound. Within an hour after withdrawal, the plasma was infused intravenously [4] into the recipients—4 euthyroid patients, 2 with thyrotoxicosis, and 1 with spontaneous myxedema. The recipient's plasma was analyzed repeatedly at intervals for 23 to 70 hours after infusion for total and protein-bound I^{131} according to methods previously described (13).

The data were subjected to standard graphic analysis, as indicated in Figure 1. Counts per minute per cc. of plasma were plotted as ordinate on semi-logarithmic paper against time following infusion as abscissa. The resultant curve could be resolved into straight line components, (*1*) an initial steep slope which could be mathematically resolved into several components, the first one of which is shown as an example in Figure 1, and (*2*) a "slower" exponential component from which the half-times ($t_{\frac{1}{2}}$) of the disappearance of the infused material from the circulation were determined. The turnover was calculated from the value for half-time of the second or slow component and the equation for exponential decay, $A = Ao e^{-kt}$,[5] using the formula:

$$\text{Turnover rate per cent per day} = \frac{\ln 2}{t_{\frac{1}{2}}} = \frac{0.693}{t_{\frac{1}{2}}}.$$

The specific activity after infusion of the thyroid hormone was determined by division of the counts per cc. plasma at zero time as obtained by extrapolation from the slow component by the recipient's protein-bound iodine concentration in micrograms per cc.; protein-bound

[1] This investigation was supported by a research grant (A-140) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, Public Health Service.

[2] Portions of this paper were read at the Forty-fourth Annual Meeting of the American Society for Clinical Investigation, Atlantic City, N. J., May 5, 1952.

[3] Part of this study was carried out during tenure as a Damon Runyon Research Fellow.

[4] The total body radiation dosage was calculated from the data of Marinelli, Quimby and Hine (14) and Marinelli (15) according to the method of Sterling (16). The total radiation delivered by the infused I^{131} ranged from .093 to .014 R.E.P.

[5] $A = I^{131}$ tagged hormone at any time.
Ao = tagged hormone at zero time.
t = time (days).
k = fraction of hormone pool turned over per day.

TABLE I

Quantitative estimations of daily thyroid hormone turnover in man

Method	Author and Reference	Estimate
Replacement requirement of athyreotics	Plummer and Boothby (4)	300 μgm. *dl*-thyroxine
	Thompson, McLellan, Thompson and Dickie (5)	325 μgm. *dl*-thyroxine (116 μgm. hormonal I_2)
	Rosenblum (6)	180 μgm./sq.M as *l*-thyroxine
	Means (7)	100 mgm. U.S.P. thyroid
	Riggs (1)	85–90 μgm. *l*-thyroxine
Mathematical analyses	Stanbury and associates (8)	45+ μgm. hormonal I_3
		83 μgm. hormonal I_3
		195 μgm. hormonal I_3
	Stanley (9)	240 μgm. hormonal I_3
	Salter, DeVisscher, McAdams and Rosenblum (10)	180–320 μgm./sq.M as *dl*-thyroxine
Disappearance rate of I^{131} labelled hormone		
a) obtained from euthyroids	Tubiana (11)	250 μgm. hormonal I_3
b) obtained from thyrotoxics	Hamolsky, Freedberg, Kurland and Wolsky	119±11 μgm. hormonal I_3

iodine determinations were carried out by a modification of Barker's method.[6] The exchangeable thyroid hormone pool was then estimated by the principle of isotope dilution, *viz.* $\frac{\text{no. of counts injected}}{\text{specific activity cts. per } \mu\text{gm.}}$. Thyroid hormone turnover (μgm. per day) was computed from the value for the exchangeable thyroid hormone pool and the per cent turnover per day. Measurement of the circulating hormone pool was made, by a similar method, utilizing data from the initial rapid component of the curve. The magnitude of the extravascular pool was approximated by subtracting the value for the circulating pool from that of the total exchangeable pool.

[6] These measurements were carried out at the PBI Laboratory of the Massachusetts General Hospital.

RESULTS AND INTERPRETATION

The identifying data concerning the nature of the material infused, the subjects of the experiments, and the results are presented in Table II. In each instance there was an initial rapid fall in radioactivity, lasting four to five hours. This was considered to result from the distribution of labelled hormone throughout the "exchangeable pool." This period also included the disappearance of the small fraction of infused non-protein I^{131} since measurements at intervals greater than one hour following infusion revealed the radioactivity to be virtually 100 per cent protein-bound. The early

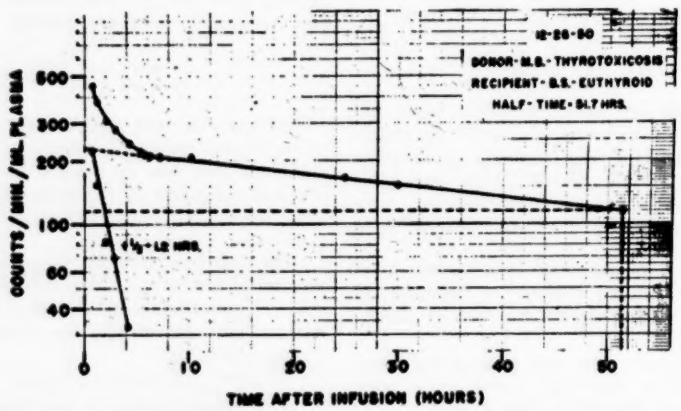

FIG. 1. DISAPPEARANCE OF RADIOACTIVITY FOLLOWING INFUSION OF LABELLED (I^{131}) HUMAN THYROID HORMONE (CASE 4)

TABLE II

Infusion of human I^{131}-labelled thyroid hormone

Case No.	Donor*			Recipient												
								I^{131} labelled hormone infused								
	Dose *mc.*	Radiation delivered to thyroid (12) *R.E.P.*	Interval between I^{131} therapy and withdrawal *days*	Clinical thyroid status	Wt. *kgm.*	Hematocrit *per cent*	Serum protein-bound iodine *μgm. %*	Volume of plasma *cc.*	Total *cts./min.*	Per cent of protein bound	Observed half-time *hrs.* (Slow phase)	Calculated turnover time *hrs.* (Half-time ×1.44)	Turnover rate *%/day*	Exchang. thyr. pool *μgm.*	Circulating thyr. pool *μgm.*	Turnover thyr. horm. *μgm./day*
1	10.6	8,040	2	Euthyroid	61.5	36	5.8	53	447,320	80	64.5	93	25.7	432	191	111
2	10.3	11,200	5	Euthyroid	66.4	40	3.9	56	1,067,000	94	50.2	72.3	33.2	323	134	107.3
3	23.1	4,965	4	Euthyroid	65.9	40	4.7	70	2,620,000	81	56.5	81.4	29.5	418	193	123.4
4	12.1	11,600	5	Euthyroid	52.2	38	5.6	50	1,725,000	95	51.7	74.4	32.2	422	176	136
Average											55.7±5.6	80.3±8.1	30.2±2.9	399±44	174±23	119±11
5	19.3	14,300	4	Thyrotox.	46.5	39	17.3	55	3,220,000	97	39.5	56.9	42.0	1142	557	480
6	9.1	5,500	6	Thyrotox.	55.9	38	11.7	70	1,771,000	96	34.0	48.9	48.8	648	280	317
7	12.9	15,250	4	Myxedema	64.6	40	1.0	48	1,350,000	96	41.2	59.4	34.3	65	23	26.2

* Each donor had thyrotoxicosis.

component was followed by a slower exponential decrease in radioactivity which has been attributed to the replacement within the exchangeable pool of the I^{131} labelled hormone by non-labelled hormone, *i.e.* the turnover of the hormone.

The half-times of the euthyroid patients were greater (50.2–64.5 hours) than those of the thyrotoxic recipients (39.5 and 34.0 hours) and the turnover rates, therefore, correspondingly less (25.7–33.2 per cent per day vs. 42 per cent and 49 per cent per day). The half-time in the myxedematous patient (41.2 hours) was shorter than that observed in the euthyroid patients. The exchangeable thyroid hormonal pool and the daily turnover of thyroid hormone were 1.5 to three times greater in the thyrotoxic patients than in the four euthyroid subjects (Table II). Values in the four euthyroid patients were in close agreement; the exchangeable pool ranged from 323 to 432 μgm. and the daily turnover from 107 to 136 μgm. Corresponding values for the patient with myxedema were strikingly less, namely 65 μgm. and 26.2 μgm./day.

DISCUSSION

The method used in the present study is based on the generally accepted concept that administered I^{131} is taken up by the thyroid gland, incorporated therein into the thyroid hormone complex and released into the circulation, thus serving as a source of endogenously labelled hormone. Thyrotoxic patients were selected as donors for the present study because in previous studies virtually complete protein-binding has been demonstrated after therapeutic doses of I^{131}. Related studies in this laboratory on several patients with thyrotoxicosis who received therapeutic doses of 5 to 20 millicuries have uniformly revealed that the protein precipitable plasma radioactivity at the time intervals used in this study is virtually all butanol soluble, *i.e.*, thyroxine-like (17). Using chromatographic and isotopic methods, Rosenberg (18) found, 2 to 10 days after administering 10 to 15 millicuries to 6 thyrotoxic patients, that practically the total organic iodine plasma radioactivity was in the thyroxin fraction which was considered to represent the circulating thyroid hormone. We have assumed, therefore, that the radioactivity of the in-

fused plasma in our cases represented natural thyroid hormone labelled with I^{131}.

Recent studies by Gross and Pitt-Rivers show clearly that the blood organic iodine labelled by I^{131} consists principally of thyroxine with smaller amounts of at least one other compound, probably triiodothyronine (19). Other questions concerning the stability of thyroid hormone endogenously labelled by I^{131}, as well as the biologic identity of thyrotoxic and euthyroid thyroid hormone (generally considered to be identical), are under investigation.

The general validity of the principles of specific activity and isotope dilution as utilized herein has been discussed by Zilversmit, Entenman, Fishler, and Chaikoff (20) and others (16, 21). Taurog, Chaikoff and Entenman (22) based their estimation of the turnover rate of protein-bound iodine in dogs on a study of the disappearance rate of pooled radioactive rat plasma (10 to 12 rats) for the first six hours following its infusion into dogs. In the course of studies on the fate of radioactive *l*-thyroxine in humans, Myant and Pochin (23) infused into 3 euthyroid patients heparinized plasma obtained from thyrotoxic patients three days after receiving up to 10 millicuries I^{131}. The plasma radioactivity was shown to have the same degree of butanol extractability as following tracer doses. They concluded "that natural thyroxine leaves the circulation more slowly than sodium thyroxine." This is in agreement with our observations regarding the early (four to six hours) portion of the curve of disappearance. Their published results (23) do not permit comparison with our data on the later component of the curve of disappearance which has been considered to represent turnover of thyroid hormone. In studies independent of those reported here, Tubiana (11) infused into 6 euthyroid subjects dialysed plasma from euthyroid humans previously treated with 50 to 100 millicuries I^{131}. The disappearance rate was followed from 6 to 48 hours. In good agreement with the observations reported here, Tubiana obtained turnover (renewal) times of 29 to 50 hours. By assuming a value of 10 μgm. per 100 cc. for plasma protein-bound iodine, Tubiana estimated an average daily hormone secretion of 250 μgm. in euthyroid subjects. Utilizing determined serum protein-bound iodine levels, the average value obtained in euthyroid subjects in the present study was 119 ± 11 μgm. per day. These results are in substantial agreement with the estimates obtained by other methods (Table I).

The size of the exchangeable pool as measured in this study is smaller than the approximation of the total body pool estimated by other methods. It is possible that the slow component represents a maximal turnover rate and that extension of the observations beyond 70 hours would demonstrate a slower component. Studies in dogs have shown an increase in serum radioactivity from 24 to 48 hours after infusion of labelled hormone attributed to the addition of hormone newly labelled by I^{131} released from the infused material. The formation, endogenously, of new I^{131} labelled hormone in the recipient and its recirculation is not believed to be a significant contributing factor to the estimate of the exchangeable pool by the method reported here since thyroid I^{131} uptake was found to be extremely low during the period of observation. It is important to emphasize that this method measures the over-all utilization within a readily exchangeable compartment. In view of the demonstrated heterogeneity of the labelled thyroid hormone complex, the possibility of variations in individual tissue exchangeability and utilization to explain the observed difference in pool size cannot be excluded.

The observation of a more rapid hormone turnover in the two patients with thyrotoxicosis is consistent with the generally held opinion of an increased metabolic turnover in this disease. It raises the interesting speculation, however, that thyrotoxicosis may be characterized not only by an increased thyroid output and body pool of hormone but also by a peripheral metabolic aberration important in the pathologic physiology of the disease. Further studies are necessary to elucidate this possibility.

SUMMARY

1. The turnover rate of human thyroid hormone has been studied by following the disappearance of plasma I^{131} obtained from thyrotoxic patients who had received 10.3 to 23.1 millicuries of radioactive iodine and infused into 4 euthyroid patients, 2 with thyrotoxicosis and 1 with myxedema.

2. The second or slow exponential decrease in radioactivity has been considered to represent the replacement of tagged hormone or its turnover.

3. The half-times in euthyroid subjects were 50.2 to 64.5 hours, in contrast to 39.5 and 34.0 hours in the patients with thyrotoxicosis and 41.2 hours in the myxedemic patient. The turnover rates were 25.7–33.2 per cent per day in the euthyroids vs. 42 and 49 per cent per day in the thyrotoxics and 34 per cent per day in the myxedematous patient.

4. The exchangeable and circulating hormonal pools have been estimated by the principle of isotope dilution and from the former the daily turnover has been calculated.

5. The exchangeable hormone pool (μgm.) averaged 399 ± 44 in the 4 euthyroid subjects vs. 1142 and 648 in the thyrotoxic patients and 65 in the myxedematous patient.

6. The daily turnover (μgm. per day) averaged 119 ± 11 in the 4 euthyroid subjects vs. 480 and 317 in the thyrotoxic patients and 26 in the myxedematous patient.

7. The method of study used permits delineation of another parameter of thyroid function; its application to varied aspects of thyroid physiology and pathology appears warranted.

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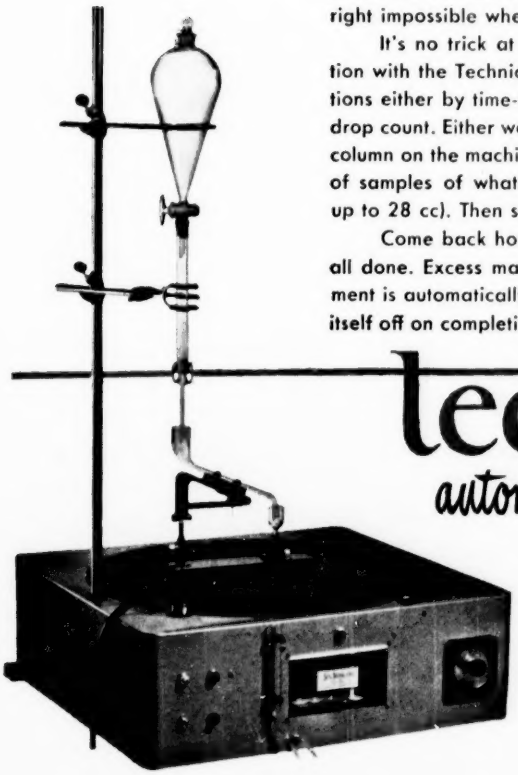

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